STUDIES ON THE DOPAMINE NEURONES IN THE SUBSTANTIA NIGRA OF THE RAT

by

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The work presented in this thesis has been composed by myself with the following exceptions.

(a) The estimations of HVA, DOPAC and GABA, described in Chapter V, were performed by Mr. N. M. Nicolaou.

(b) Estimations of striatal DA concentration, also described in Chapter V were performed by Mrs. A. Wright.

(c) Electrolytic lesioning of the striato-nigral pathway was performed by Mrs. M. Garcia.

Signed,

(I. F. Tulloch, B.Sc.)

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SUMMARY

1. Single, spontaneously active neurones have been recorded extracellularly in the substantia nigra of the halothane-anaesthetised rat.
2. On the basis of electrophysiological, histological and pharmacological criteria, and in agreement with the reported literature, neurones in the zona compacta region and ventral tegmental area were identified as being DA-containing.
3. The responsiveness of identified nigral DA neurones to electrical stimulation of known brain olfactory areas was tested. Whilst stimulation of the ipsilateral habenular nucleus and olfactory bulb proved ineffective in influencing DA cell firing rate, it was found that relatively strong electrical stimulation of the ipsilateral anterior olfactory nucleus evoked long latency, complex responses from these neurones. It could not be determined whether these were responses to a specific olfactory input or a non-specific arousal.
4. A degeneration study revealed that the habenular nucleus does not project directly to the region of the DA-containing cells in the SN. Further anatomical studies indicated that medial forebrain bundle (MFB) path neurones, located in the lateral hypothalamus may be involved in relaying olfactory information to the SN.
5. Intra-nigral injections of HRP, well localised to the zona compacta region, further supported the possibility that MFB path neurones project to this region. In addition HRP-labelled cells were observed in the dorsal raphe nucleus, in the region of the

superior cerebellar peduncle, paraventricular nucleus, tail of the striatum, globus pallidus and interstitial nucleus of the stria terminalis.

6. The entire course of the striato-nigral pathway and its termination in the zona reticulata region were plotted autoradiographically. Rats with unilateral lesions in this pathway were found to turn towards the lesioned side when treated with apomorphine (0.5mg./kg. i.p.) or amphetamine (2mg./kg.i.p.). These lesions also caused significant decreases (40-50%) in nigral GABA concentration but failed to significantly alter the basal or haloperidol-stimulated DA metabolism in the ipsilateral striatum. It was suggested that the striato-nigral pathway does not function as a "feedback pathway" controlling the firing rate of DA neurones projecting to the striatum.

CHAPTER ONE

GENERAL INTRODUCTION

Sympathin, a substance with biological properties resembling those of noradrenaline (NA), was first detected in mammalian brain tissue in 1947 by von Euler. Subsequently it was shown that sympathin was predominantly NA with a small admixture of adrenaline (Holtz, 1950). Further analysis revealed that it was clearly unevenly distributed in the brain with the highest concentrations being found in the hypothalamus and hind brain (Vogt, 1954). Later biochemical studies indicated that a third catecholamine compound was present in brain tissue (Montagu, 1957) and on the basis of its chromatographic properties this amine was suggested to be 3-hydroxytyramine or dopamine (DA). This was confirmed by Carlsson, Lindqvist, Magnusson and Waldeck, 1958, using an improved fluorometric technique. These workers also reported that an elevation of brain DA levels occurred following the administration of l-dopa. The behavioural effect accompanying this rise in brain DA (and NA) was noted as a "central stimulation".

The first detailed biochemical mapping of DA and NA in brain tissue (Bertler and Rosengren, 1959) demonstrated that the distribution of DA differed greatly from that of NA; the highest concentration being found in the basal ganglia with small but significant amounts in other brain areas, notably the mesencephalon. This evidence, plus the finding that NA and DA concentration in brain were approximately equal provided reasonable grounds for postulating that DA was not simply a precursor of NA but might by itself have an important

function; perhaps a neurohumoral one.

These biochemical findings prompted further investigation into the precise localisation of these catecholamines in the brain. Earlier work by Bränko in 1955 had provided a clue to a possible histochemical technique that could be of great potential value in this mapping work. This worker found that formalin fixation of catecholamine containing peripheral tissue e.g. the adrenal medulla, resulted in the formation of a highly fluorescent insoluble product in the NA-containing medullary islets. This fluorescence was not present in NA depleted tissue suggesting that the histochemical reaction was dependent on the presence of this catecholamine. Unfortunately NA was readily soluble in formalin and this precluded the use of the technique in demonstrating this amine in sympathetic nerves where its concentration was relatively small. The method was substantially improved by freeze-drying the tissue and exposing it to formaldehyde vapour under carefully controlled conditions of temperature and humidity (Falck 1962; Falck, Hillarp, Thieme and Torp, 1962). This had the effect of limiting diffusion of the catecholamines as well as increasing the sensitivity of the method. The technique was also extensively studied in a model system that used a dried serum albumin layer as the reaction medium (Falck, Thieme, Torp and Hillarp, 1962). This enabled the precise structural requirements of the substrates and the optimal reaction conditions for the condensation of formaldehyde and substrate to be determined. Of great importance was the finding that formaldehyde vapour formed intensely fluorescent condensation products with the catecholamines DA and NA as well as the indolealkylamine, 5-hydroxytryptamine (5HT) and that the metabolites of these monoamines

were either present in undetectable amounts in brain tissue or they did not form condensation products. Moreover, the fluorescence spectrum obtained on condensation of 5-HT with formaldehyde was distinctly displaced to a higher wavelength as compared to that of catecholamines (Falck, 1962). The 5-HT fluorescence was characterised by a yellow colour, whereas catecholamines exhibited a green to yellow-green coloration. This permitted a histochemical distinction to be made between the 5-HT and the catecholamine systems.

The initial studies on brain using the Falck-Hillarp technique centred on the hypothalamus (Carlsson, Falck, and Hillarp, 1962), and the spinal cord (Carlsson, Falck, Fuxe and Hillarp, 1964; Dahlström and Fuxe, 1964(b)). The monoamines NA and 5-HT were found to be present in highly fluorescent varicose structures that were postulated to be the terminals of noradrenergic and serotinergic neurones respectively. Pharmacological methods were also used to change the biochemically estimated brain monamine levels in predictable ways (Carlsson, Lindqvist and Magnusson, 1960; Carlsson, Falck and Hillarp, 1962), and these were used in conjunction with the known monoamine distribution to give the histochemical technique an increased specificity. Of particular importance in this respect was the methylated amino acid, alpha-methyl meta tyrosine. This compound was known to cause a transient decrease in brain 5-HT and DA stores but a prolonged and more marked decrease in NA (Carlsson and Lindqvist, 1962). The cautious use of this compound permitted a distinction to be drawn between NA and DA, both of which had identical fluorescent spectra when treated according to the Falck-Hillarp technique.

With these advances in histochemical techniques it soon became possible to systematically map out the monoamine containing neurones in the central nervous system (Dahlström and Fuxe, 1964(a), 1965). Altogether, 21 different monoamine cell groups were identified and named. The 5-HT groups were termed B groups and were numbered 1 to 9, depending on their rostro-caudal position in the brain. Similarly, the catecholamine-containing neurones were classified into 12 distinct groups and termed A groups. Groups A1 to A7 were identified as NA-containing neurones and were located in the pons and medulla oblongata regions. A8 to A10 were found in the region of the substantia nigra (SN) and ventral tegmental area (VTA) of the mid-brain and were claimed to consist of DA-containing cells. Lastly, groups A11 and A12 were found in the diencephalon and identified as DA-containing cells. It also proved possible to map out the monoamine terminal areas by making extensive use of the pharmacological methods available to selectively enhance or deplete 5-HT, NA and DA systems (Dahlström and Fuxe, 1965; Fuxe, 1965). There was little doubt that the strongly fluorescent monoamine-containing terminals observed belonged to the specific monoamine neurones since they disappeared after axotomy or destruction of the nerve cell bodies (Andén, Carlsson, Dahlström, Fuxe, Hillarp and Larsson, 1964; Andén, Dahlström, Fuxe and Larsson, 1964; Dahlström and Fuxe, 1964(b)).

On the basis of the histochemical localisation of the DA-containing cells in the SN and VTA attempts were made to precisely map out the pathways from these neurones to the proposed DA terminal areas, which were predominantly found in the caudate nucleus,

tuberculum olfactorium, nucleus accumbens and median eminence (Fuxe, 1965). The fine axons of the catecholamine neurones are normally not fluorescent when studied with the Falck-Hillarp technique but it was found that axotomy of these fibres resulted in a marked accumulation of fluorescent material proximal to the site of the lesion (Dahlström and Fuxe, 1964(c)). This was presumably due to pile-up of monoamine-containing granules which are normally transported down the neurones from the cell body to the terminals. By lesioning the caudate nucleus it was clearly demonstrated that the A9 group of cells showed a marked decrease of fluorescent intensity (Andén, Dahlström, Fuxe and Larsson, 1965). Lesions of the A9 cell group resulted in a marked loss of fluorescence in the caudate nucleus (Andén, Carlsson, Dahlström, Fuxe, Hillarp and Larsson, 1964). These lesion studies also allowed a fairly accurate localisation of the nigro-neostriatal DA pathway by making use of the proximal pile-up of fluorescent material following these lesions. However, the pathway was not shown in its entirety.

These histochemical findings were confirmed by independent biochemical studies in the monkey and cat (Poirier and Sourkes, 1965; Poirier, Singh, Boucher, Olivier and Larochelle, 1967). In both these studies it was reported that lesions placed in the VTA of the midbrain resulted in a substantial depletion of DA in the ipsilateral caudate nucleus. Further experiments showed that striatal DA synthesis was also markedly reduced following similar VTA lesions (Goldstein, Anagnoste and Owen, 1966). Electrical stimulation in the region of the SN was shown to increase DA release in the putamen (McLennan, 1964),

as well as the DA metabolite, homovanillic acid, collected in perfusates of the striatum (Portig and Vogt, 1968). The existence of this direct nigrostriatal pathway was also supported by numerous electrophysiological studies (Connor, 1968, 1970; Frigyesi and Purpura, 1967; Hull, Barnadi and Buchwald, 1970). Complete agreement on the existence of this pathway was lacking since it could not be demonstrated by the classical Nauta silver impregnation technique for degenerating pathways (Faull and Carmen, 1968). However, this was due to a limitation of this particular technique since it did not stain very fine unmyelinated fibres, such as those of the DA neurones.

The nigro-neostriatal pathway, arising from DA-containing neurones located in the A9 cell group, was certainly the most intensely investigated central monoamine system. Unfortunately, the other brain monoamine systems had been more or less neglected, largely due to the difficulty in accurately mapping out the projections of the pathways arising from the various monoamine cell groups. Investigations were hampered due to the difficulty in visualising the fine, unmyelinated axons of the monoamine neurones and the inability of the Falck-Hillarp technique to distinguish between NA and DA. However, these difficulties were gradually overcome by the painstaking use of well localised lesions as well as various biochemical techniques (Ungerstedt, 1971). By using a series of brain hemi-sections Ungerstedt was able to identify the monoamine pathways at various brain levels due to proximal pile-up of monoamines in the severed axons. Thereafter, electrocoagulation and specific degeneration of catecholamine-containing axons and cell bodies by intracerebral

injections of 6-hydroxydopamine (6-OHDA) revealed a wealth of data on the monoamine systems. Ungerstedt complemented his extensive lesion work with biochemical studies, using alpha-methyl-NA. This compound, when injected into brain areas rich in catecholamine terminals, is taken up by these terminals and transported retrogradely along the axons. It was found that this compound reacted strongly when treated according to the Falck-Hillarp reaction conditions and it accumulated in a sufficient concentration in the axons to permit the entire catecholamine pathway to be visualised. Partial lesions of the monoamine cell groups, identified previously by Dahlström, and Fuxe, 1964(a), in particular the DA cells in the SN and VTA, allowed Ungerstedt to plot the topographical projections of these cells. He concluded that the A8 and A9 groups projected to the corpus striatum, whereas the A10 group, via a more medial pathway, innervated the nucleus accumbens and the tuberculum olfactorium.

Recent improvements in the histochemical fluorescent method for catecholamines have been reported (Lindvall and Björklund, 1974), which make use of glyoxylic acid rather than formaldehyde vapour. The published results by these workers on the distribution of catecholamines agree to a large extent with those of Ungerstedt. The glyoxylic method is more sensitive than the Falck-Hillarp technique and it is possible to visualise the entire catecholamine axons without recourse to drug treatment or lesioning. Moreover, it is possible to differentiate between NA and DA systems on the basis of the morphological differences that exist between DA axons and terminals and those

of NA. Although Lindvall and Björklund, 1974, suggested the same projections for the A8 and A9 groups as Ungerstedt, they further stated that the A10 cell axons ramified through the forebrain and terminated in the limbic and frontal cortices in addition to the mesolimbic areas.

Biochemical studies on DA synthesis in these cortical regions supported the possibility of dopaminergic terminals being present in these areas (Thierry, Blanc, Sabel, Stinus and Glowinski, 1973; Berger, Tassin, Blanc, Moyne and Thierry, 1974). The origin of these cortical dopaminergic terminals appears to be the medial A9 and the lateral A10 cell group (Fuxe, Hökfelt, Johansson, Lidbrink and Ljungdahl, 1974). Following lesions in this area it was found that the dopaminergic terminals completely disappeared from the entorhinal, amygdaloid and frontal cortical areas. Thus, the SN DA system ramifies more widely than was originally realised and it is possible that other pathways exist but remain undetected with the present histochemical techniques.

In view of the tremendous amount of reported work on the SN dopaminergic systems it is surprising to find that so little is known about the neural projections to this area. In the present study the overall aim has been to investigate this problem by using a combined electrophysiological and neuro-anatomical approach.

The major section of the experimental work is electrophysiological in nature and involves single cell, extracellular recording

of identified nigral DA neurones. In Chapter II the electrical characteristics of the SN neurones are described in detail with particular emphasis on the DA neurones. Histological and pharmacological criteria used in the identification of the DA neurones are also described.

The fact that DA cells could be identified electrophysiologically permitted a further study into the responsiveness of these neurones to electrical stimulation of other brain areas thought to be important in influencing DA cell activity. This study is described in Chapter III. In addition, the electrophysiological approach was complemented by anterograde tracing techniques, including the radioactive leucine method (Cowan, Gottlieb, Hendrickson, Price and Woolsey, 1972) in a bid to map out possible afferent pathways to the nigral DA system.

To further investigate afferent projections to the SN use has been made of the retrograde tracing technique, utilising intracerebral injections of horseradish peroxidase. This technique is described in detail in Chapter IV. Intra-nigral injections of this enzyme were performed and the resulting distribution of retrogradely labelled cells is also described in detail.

The autoradiographic tracing technique, described in Chapter III was further employed to map out the efferent pathways of the caudate nucleus; in particular the course and projection of the striato-nigral pathway. This neuroanatomical study formed the basis of Chapter V. It proved possible to separate anatomically the nigro-

striatal DA system from the striato-nigral system, thus enabling the former system to be studied biochemically, following destruction of the striato-nigral pathway.

CHAPTER II

IDENTIFICATION OF NEURONES IN THE SUBSTANTIA NIGRA USING A COMBINED ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL APPROACH

CHAPTER TWO

INTRODUCTION

Carlsson and Lindqvist in 1963 first suggested that the two antipsychotic drugs, chlorpromazine and haloperidol could possibly block monoamine receptors in the central nervous system. They found that these two drugs had no effect on brain dopamine (DA) and noradrenaline (NA) concentrations, but that administration of either drug, after monoamine oxidase inhibition, resulted in a marked increase in 3-methoxytyramine and normetanephrine, the O-methylated metabolites of DA and NA respectively. The hypothesis forwarded was that these drugs acted by blocking monoamine receptors and this blockade led to a compensatory activation of the monoamine neurones by some kind of neuronal "feedback" mechanism. In order to explain the lack of effect of chlorpromazine and haloperidol on the brain DA and NA concentrations, Carlsson and Lindqvist further suggested that this increase in monoamine neuronal activity resulted in a stimulation of monoamine synthesis in these neurones.

This hypothesis stimulated a great deal of research into the mode of action of antipsychotic drugs, especially the phenothiazines and butyrophenones. Numerous methods were developed to estimate the turnover of catecholamines in the brain and in general it has been found that these antipsychotic drugs increase DA turnover to a greater extent than they do NA turnover (Anden, Butcher, Corrodi, Fuxe, and Ungerstedt, 1970; Keller, Bartholini, Pletscher, 1973; Berridge, and Sharman, 1974). Following inhibition of tyrosine hydroxylase by alpha methyl para tyrosine (AMPT) chlorpromazine increased the rate of

disappearance of DA (Corrodi, Fuxe and Hökfelt, 1967). This drug also increased the rate of formation of DA from labelled tyrosine (Nyback and Sedvall, 1968) and the brain concentration of homovanillic acid (HVA), an acidic metabolite of DA (Da Prada and Pletscher, 1966). Thus, all the later biochemical evidence supported the original hypothesis of Carlsson and Lindqvist but it did not throw much light on the mechanism whereby these antipsychotic drugs increased DA metabolism.

The brain catecholamine mapping work (see General Introduction for references) permitted studies to be carried out on the identified DA systems, in particular the nigro-striatal pathway. Acute lesions placed in the region of the DA cell bodies in the SN were reported to completely abolish the facilitatory effects of chlorpromazine and haloperidol on ipsilateral striatal DA metabolism (Andén, Corrodi, Fuxe and Ungerstedt, 1971; Nyback and Sedvall, 1971). This suggested that nervous impulse flow in the nigro-striatal system was essential for these drugs to increase striatal DA metabolism and partly resolved the problem of whether the "feedback" mechanism was chemical or nervous in nature.

The negative "feedback" hypothesis, as originally proposed, was not supported by any experimental results following an enhanced stimulation of dopaminergic receptors, because no suitable DA receptor agonist was known at that time. However, it was later shown from behavioural studies that the drug apomorphine could mimic the compulsive gnawing syndrome produced by the intraperitoneal

administration of the DA precursor l-dopa (Ernst, 1965). Moreover, intracerebral injections of apomorphine and l-dopa also produced this syndrome but only when injected into the corpus striatum (Ernst and Smelik, 1966). Apomorphine, when administered intraperitoneally could still elicit this gnawing behaviour, even after DA depletion with AMPT (Ernst, 1967). This evidence, in addition to the structural similarities between apomorphine and DA further suggested that this characteristic behaviour resulted from a direct effect on striatal DA receptors and not by the release of endogenous DA.

Apomorphine, in contrast to chlorpromazine and haloperidol, caused a retardation of the depletion in brain DA following the administration of H44/68, a potent inhibitor of tyrosine hydroxylase (Andén, Rubenson, Fuxe and Hökfelt, 1967). Furthermore, this action of apomorphine was blocked by haloperidol suggesting that both these drugs were acting at the same receptor site. This evidence extended the negative feedback idea to include the action of DA agonists and that enhanced stimulation of DA receptors decreased the activity of DA neurones. As further supportive evidence of this feedback inhibition of the DA system, it was reported that apomorphine caused a significant decrease in HVA concentration in the striatum (Roos, 1969).

The possibility of stimulating DA receptors by causing the release of endogenous DA presented another way of testing this feedback hypothesis. It had been reported that d-amphetamine released extragranular DA (Carlsson, Fuxe, Hamberger and Lindqvist, 1966) in

nialamide pre-treated rats. Later evidence from studies on the perfused, isolated rat striatum (Besson, Cheramy, Feltz and Glowinski, 1969) and the intact cat striatum (Besson, Cheramy, Feltz and Glowinski, 1971) showed that d-amphetamine caused a marked enhancement of DA release from this nucleus. The released DA was known to have been synthesised from the labelled precursor, tyrosine, thus strongly indicating that the DA was indeed being released from striatal DA terminals and not from some extraneuronal store. If d-amphetamine did release endogenous DA then it was reasonable to predict that this drug would decrease brain DA levels following enhanced stimulation of DA receptors. However, after tyrosine hydroxylase inhibition by H44/68, amphetamine in low doses, did not alter the rate of depletion of DA in whole brain (Corrodi, Fuxe and Hökfelt, 1967). This result was explicable in terms of the feedback hypothesis if it was supposed that amphetamine caused an increased amount of DA to reach the DA receptors at post-synaptic sites (i.e. not located on DA neurones) and this resulted in a negative feedback onto the DA neurones. This reduction in impulse flow would presumably decrease the release of DA from the terminals and counter the loss caused locally by the amphetamine.

Although the above biochemical evidence strongly supported Carlsson and Lindqvist's original hypothesis, it has been recently discovered that a local regulation of striatal release and synthesis exists which is independent of DA neurone impulse flow (Farnebo and Hamberger, 1971). Using a preparation of striatal slices, electrically stimulated by constant frequency field stimulation, these workers showed that apomorphine caused a slight decrease of the stimulation-

induced overflow of DA, whereas the DA antagonists pimozide and chlorpromazine increased this overflow. In this preparation there was presumably no intact neuronal feedback loop and the effect was independent of impulse flow since this was kept constant, albeit rather artificially. Although the observed biochemical effects of DA antagonists had been reported to be secondary to changes in nerve impulse frequency in the DA neurones (Anden et al., 1971; Nyback and Sedvall, 1971) these in vitro results indicated that this was perhaps not the entire story and that a more local effect on DA release was also an important regulatory mechanism.

Evidence for this local control of DA release has also been reported from studies involving lesioning of the nigro-striatal system. These lesions cause a doubling of the endogenous DA concentration in the ipsilateral striatum (Anden, Bedard, Fuxe and Ungerstedt, 1971) which was due to an activation of the enzyme tyrosine hydroxylase (TOH) (Carlsson, Kehr, Lindqvist, Magnusson and Atack, 1972) estimated by following dopa synthesis. This increase in synthesis is transient in nature, lasting only approximately 1 hour after axotomy. If apomorphine was administered prior to lesioning then this elevated dopa synthesis did not occur. It was also reported that haloperidol abolished this blocking effect (Carlsson et al., 1972) suggesting that a DA receptor mechanism might be important. Undoubtedly the overriding problem with this work was in the understanding of how it was possible that TOH could be activated in the presence of increased DA concentration, since the favoured theory at that time was that when intraneuronal catecholamine concentrations increased then this resulted in end-product inhibition of TOH (Levitt,

Spector, Sjoerosma and Udenfriend, 1965). Carlsson et al., 1972 proposed that perhaps a presynaptic DA receptor was responsible for the observed effects. Interuption of impulse flow, it was suggested, would presumably decrease the concentration of DA in the synaptic cleft, leading to a reduction in presynaptic DA receptor activation. By a mechanism that was not at all clear this was thought to be important for the activation of TOH.

More detailed studies on the mechanism of activation of TOH (Roth, Walters, Murrin and Morgenroth, 1975) showed that after axotomy of the nigro-striatal system, this enzyme had a marked increase in its affinity for tyrosine and pterin cofactor and a large decrease in its sensitivity to end-product inhibition by DA. These workers also reported that addition of Ca^{++} ions to the enzyme incubation medium completely abolished the observed activation of TOH and suggested that CA^{++} fluxes across the DA neuronal membrane may be very important in the regulation of TOH.

This response of DA neurones after axotomy seems to be unique in the central monoamine systems since the 5-HT and NA systems do not show this increase in transmitter synthesis after acute lesions in the midbrain raphe nuclei and locus coeruleus (Carlsson et al., 1972; Korf, Aghajanian and Roth, 1973). However, lesions of the septohippocampal cholinergic pathway have been shown to result in an increase in the levels of endogenous acetylcholine (ACh) (Sethy, Kuhar, Roth, van Woert and Aghajanian, 1973).

Increase in the impulse flow in the nigro-striatal system by

direct electrical stimulation had also been found to cause a stimulus-dependent increase in striatal dopa synthesis but without changing the levels of endogenous DA (Murrin and Roth, 1973; Roth, Walters, Murrin and Morgenroth, 1975). This has been found to be a very similar activation of TOH to that observed after cessation of impulse flow (Murrin, Morgenroth and Roth, 1974). Similar findings have been reported in the peripheral and central noradrenergic systems (Morgenroth, Boadle-Biber and Roth, 1974; Salzman and Morgenroth, 1974). Enhanced neuronal activity in all these systems results in an increased affinity of TOH for its substrate tyrosine and pterin cofactors and a decreased affinity for the natural end-product inhibitor (NA or DA). The situation is thus more complex than increased impulse flow causing a reduction in intraneuronal DA and consequently removing end-product inhibition. Similar kinetic effects on TOH can also be observed when cyclic AMP is added to in vitro striatal preparations that contain TOH (Harris, Baldessarini and Morgenroth, 1974). This has led to the speculation that there may be a presynaptic adenylate cyclase system which is activated during impulse flow (Roth, Walters, Murrin and Morgenroth, 1975).

The stimulation induced increase in TOH can be modulated by DA agonists and antagonists in a similar way to that which occurs following cessation of impulse flow. Administration of the DA agonist ET495 caused a significant reduction in the induced activation and antipsychotic drugs, such as haloperidol and chlorpromazine cause an increase (Roth et al., 1975). It was assumed that receptor activation by a DA agonist results in a braking effect that counteracts the stimulus-induced activation of TOH. In these experiments the electrical

stimulation was such that the TOH was activated to a maximal extent and a blockade of post-synaptic DA receptors by drugs would not be expected to increase the impulse rate in the DA neurones via a feedback pathway. Thus, the involvement of a presynaptic DA receptor has been proposed (Roth et al., 1975). The mechanism of stimulus-induced activation is not known but it does seem to be different to that which occurs after cessation of impulse traffic in the pathway since Ca^{++} ions, when added in vitro to the TOH preparation, do not inhibit the stimulation-induced enzyme activation.

Although these "presynaptic" effects could be explained by the DA agonist and antagonist drugs acting post-synaptically on DA receptors it would require some kind of transsynaptic mediator to modulate the DA synthesis and release from the DA neurone terminals. Thus, the simpler idea of a presynaptic DA receptor, or autoreceptor, has gained much support. However, the fundamental problem has been in separating these possible pre- and post-synaptic effects from each other and investigating their roles in the normal functioning of the DA neurones in the SN. Carlsson has recently proposed that the biphasic effect of apomorphine on dopa synthesis in the striatum is due to activation of two different types of DA receptor (Carlsson, 1975). Apomorphine inhibits synthesis after low doses and then there is a plateau followed by a further decrease with higher doses. He suggested that the first phase was due to activation of autoreceptors and the second phase was due, at least partly, to post-synaptic receptor activation. This evidence in support of the two receptor hypothesis is somewhat tenuous, however, and can only be regarded as speculative at the moment. Nevertheless, it does seem likely that the

control of DA cell firing rate, metabolic activity and DA release are controlled by at least two and perhaps three different independent receptor mechanisms.

ELECTROPHYSIOLOGY OF THE SUBSTANTIA NIGRA DA NEURONES

The DA-containing cells in the zona compacta region of the SN and the VTA were first recorded extracellularly by Bunney, Walters, Roth and Aghajanian in 1973. These workers placed special emphasis on the identification of this very small group of neurones and reported that it was not possible to record from the presumed DA cells following selective degeneration of the DA-containing cells by intranigral administration of 6-OH DA. The electrophysiological characteristics of the zona reticulata neurones were unaffected by this treatment. This experiment strongly supported their claim that they were indeed recording from DA-containing cells and, short of marking a recorded cell intracellularly with a dye, provides the most convincing evidence to date.

The most characteristic electrophysiological properties of the DA-containing cells, reported by these research workers, was that they had a comparatively slow firing rate and tended to display an occasional burst-like activity. The firing frequency and pattern did seem to depend on the anaesthetic employed e.g. under chloral hydrate the cells exhibited a marked burst-like activity that was not present in gallamine-paralysed animals. The firing rate also increased with this anaesthetic. Obviously these changes in DA cell activity with various anaesthetics create problems that can only be overcome by employing reliable identification criteria.

Fortunately, the pharmacological effects of systemically administered DA agonist and antagonist drugs on DA cells do seem to be independent of the anaesthetic used but it is perhaps premature to make this a general statement since the literature indicates that most work has been performed on gallamine-paralysed rats (Bunney et al., 1973(a)) or tubocurarine-paralysed rats (Rebec and Groves, 1975) with chloral hydrate and halothane being the only reported anaesthetics used (Bunney et al., 1973(a)). This latter group of workers reported that the DA cells responded to DA receptor stimulation or blockade in a similar manner in both the paralysed and anaesthetised animals.

Apomorphine, d-amphetamine and l-dopa when administered intravenously in small doses, selectively decrease the firing rate of identified DA neurones (Bunney, Aghajanian and Roth, 1973; Bunney, Walters, Roth and Aghajanian, 1973). This effect of d-amphetamine, in contrast to that of apomorphine, was abolished by pre-treatment with alpha methyl para tyrosine, thus indicating that continuing DA synthesis was necessary for amphetamine to produce this effect. In addition, the inhibition of DA cell firing rate observed with these three drugs was blocked and reversed by various antipsychotic drugs, including haloperidol and chlorpromazine (Bunney, Aghajanian, and Roth, 1973). These postulated DA receptor blocking drugs, when administered alone, markedly increased the firing rate of the DA cells (Bunney et al., 1973(a)).

Thus, the early electrophysiological work on the nigral DA cells supported the concept of a neuronal feedback pathway. The major problem,

as in the biochemical approach, was in establishing whether these effects were mediated through pre- or post synaptic DA receptors. All the effects so far mentioned could be explained by postulating that pre-synaptic DA receptors were the site of action of these drugs, thus making the idea of a feedback loop rather unnecessary. However, Bunney and Aghajanian, 1973, reported that transections placed at a level just anterior to the SN abolished the depressant effect of systemically-administered d-amphetamine on the DA cells, as well as increasing their basal firing rate. These lesions were claimed to interrupt the striato-nigral pathway which was suggested by these workers to be the neuronal feedback pathway, via which amphetamine exerted its depressant effect on DA neurones following DA release and DA receptor stimulation in the caudate nucleus. These transections were relatively crude and they would also have cut the nigro-striatal pathway since these two pathways run in close proximity to each other (Tulloch, 1976). Recently this transection study has been further criticised on the grounds that amphetamine-induced release of DA from dopaminergic terminals in the striatum is blocked by acute lesions placed in the nigro-striatal pathway (Groves, Wilson, Young and Rebec, 1975). Dopaminergic neurones in these transected preparations were readily inhibited by relatively low doses of apomorphine and this effect reversed by intravenously administered haloperidol. This evidence further supported the concept of a pre-synaptic DA receptor located on the DA neuron, since this animal preparation had no intact striatal efferent pathway that projected to the SN and through which post-synaptic DA receptor activation in the striatum could influence DA cell activity in the SN.

Microiontophoretic studies on the SN DA cells have also supported a pre-synaptic mode of action for DA agonist drugs (Bunney and Aghajanian, 1973). These workers reported that DA cells in the zona compacta region and VTA were markedly depressed by iontophoretically applied DA and apomorphine, and suggested that there may be DA receptors on the soma of the DA neurones. In support of this is the fact that systemically administered haloperidol or chlorpromazine blocked this effect (Bunney and Aghajanian, 1975). The importance of these postulated receptors in normal function is unknown but there are some similarities between the iontophoretic effects of DA and apomorphine on DA neurones and those reported after systemic administration of these drugs. In each case there is a marked initial depressant effect on the neurones which diminishes upon subsequent administration of the drugs (Aghajanian and Bunney, 1973; Walters, Bunney and Roth, 1975).

These marked depressant effects are in contrast to the very weak and transient depressant effect of iontophoretically applied d-amphetamine (Bunney and Aghajanian, 1973). There was a rapid diminution of the response with repeated application of the drug and it was not considered to be of physiological significance. However, d-amphetamine was found by the same researchers to strongly depress the firing rate of neurones in the striatum and this effect showed no diminution upon repeated iontophoretic application. This was further evidence that systemically administered d-amphetamine was unlikely to depress DA neurones by a direct action but perhaps acted indirectly via the striato-nigral pathway.

INTRACEREBRAL ADMINISTRATION OF DRUGS AFFECTING DA CELL ACTIVITY

From the preceding biochemical and electrophysiological results it is clear that at least three anatomically distinct DA receptors have been postulated to exist in the brain. This makes interpretation of the effects of systemically administered drugs particularly difficult and in an attempt to separate out these receptor effects on DA cell firing rate use has been made of the intracerebral route of administration (Groves et al., 1975). In support of the postulate that DA cell somata have DA receptors these workers reported that intra-nigral injection of d-amphetamine markedly depressed the firing rate of zona compacta cells with concomitant stimulation of striatal neurones. These results are in contrast to the lack of effect of microiontophoretically-applied d-amphetamine on these neurones (Bunney and Aghajanian, 1973(a)). In addition, the latter research group claimed that intravenously administered d-amphetamine failed to depress DA cell firing in preparations transected in the diencephalon as previously described.

It has been argued that DA cell dendrites may possibly release DA and thus self-inhibit other dopaminergic neurones (Groves et al., 1975). These workers also suggested that d-amphetamine released this dendritic store of DA, but it is unclear as to why this effect should occur only after intra-nigral administration of this drug and not after microiontophoretic administration. Groves et al., 1975, further reported that intra-striatal injections of amphetamine resulted in an increase in DA cell firing rate, a finding consistent with a positive feedback function for the striato-nigral "feedback" pathway. Haloperidol administered in a similar manner inhibits DA

cell firing rate. These data are certainly difficult to reconcile with the idea of a negative "feedback" pathway controlling DA cell firing rate. Perhaps one explanation for these seemingly contradictory results is that the dopaminergic cell self-inhibition may override a positive feedback from the striatum and thus the overall observed effect is a negative feedback one.

With this pharmacological and electrophysiological background in mind an attempt has been made in this present study to identify and distinguish electrophysiologically neuronal populations in the SN. Three principal criteria were used in this identification. Firstly, the recording sites in the SN were verified histologically, in both stained and Falck-Hillarp reacted sections. Secondly, the electrical characteristics of the histologically identified neurones were compared with those reported in the literature. Lastly, a selection of cells fulfilling the first and second criteria were tested for their responsiveness to intravenously administered DA agonist and antagonist drugs. In some experiments the blood pressure and respiratory rate of the preparation were monitored.

METHODS

1. Falck-Hillarp Technique for Visualising Brain Catecholamine

The technique used was essentially similar to that described by Dahlström and Fuxe, 1964.

Experimental rats (n=6) were stunned and quickly decapitated and the brain was rapidly dissected out and placed on an ice-cold tile. Small pieces of brain tissue less than 5mm in thickness and containing the DA containing cells in the substantia nigra, were carefully dissected and placed on stiff, pre-labelled pieces of cardboard. These were then quickly immersed in isopentane that had been cooled with liquid nitrogen (Falck and Owman, 1965). The time interval between killing and freezing the tissue must be kept to a minimum to reduce the post-mortem diffusion of catecholamines from neurones. In the following experiments the time interval was less than four minutes. Dahlström and Fuxe used liquid propane as a freezing agent but it was found that isopentane gave as good results. The frozen tissue was then transferred to a metal tray which had been pre-cooled in liquid nitrogen and thereafter placed in the tissue compartment of a freeze-drier (Olson and Ungerstedt). This compartment contained two large trays of phosphorus pentoxide, used as the drying agent. The compartment was cooled to a temperature of -35°C and evacuated to a pressure less than 0.01 Torr.

After approximately three days freeze drying the tissue was allowed to warm up to 25-28°C and then quickly transferred to a 1 litre desicator containing 5gm of paraformaldehyde, stored

previously at a relative humidity of 71%. The desicator was placed in an oven and maintained at 80°C for 1 hour. These reaction conditions resulted in the formation of the catecholamine fluorescent products, discretely localised in the DA neurone cell bodies and terminals.

Following this reaction step the tissue was vacuum-embedded in paraffin wax (M.P. 54°C) for a period of 10 minutes. Thus treated the tissue could be stored for some weeks. The wax blocks were then mounted on a microtome and sections of 10μm thickness were collected and mounted on pre-heated glass slides (60°C). After the sections had melted onto the slides they were allowed to cool before mounting in a medium containing 0.04ml xylene per 10ml of Entellan mountant. These slides were stored in darkness to prevent fading of the fluorescent products and were usually examined within 1 day of mounting, in a Zeiss "Universal" microscope. The light source was a high intensity HB0200 W/4 super pressure mercury lamp and the sections were viewed under dark-field illumination. A 3mm BG-12 excitation filter was used in conjunction with a K500 barrier filter.

2. Etching and Insulation of Tungsten Microelectrodes.

(a) Etching

The electrodes selected in the following experiments were tungsten microelectrodes first described by Hübel in 1957. These particular electrodes have the advantage that they are relatively robust and easy to make but suffer from the disadvantage that they are difficult to make consistently well.

Short pieces of straight, pre-cleaned tungsten wire (Tungsten Manufacturing Co.) with a diameter of 0.01 inches were fixed in pin chucks, which were then mounted onto the frame of a dipping device. The etching solutions chosen were saturated NaOH and 20M $NaNO_2$. The initial etching procedure involved partially immersing the electrodes into the NaOH solution and passing a 6V a.c. current through them, using a carbon rod as the indifferent electrode. This resulted in the electrodes having a uniform length. Thereafter, the electrodes were repeatedly dipped into this solution until they became as sharp as possible, usually 3-5μm. Following this step the $NaNO_2$ solution was substituted for the NaOH and the etching current was decreased to approximately 3V a.c. Slow, even dipping, repeated 5-10 times resulted in electrodes having tip diameters of 1μm or less. This type of electrode tapered gently to a very fine point. In some cases electrodes having a pencil-like tip were made as reported originally by Hubel. The electrodes were etched to a long, very fine tip in $NaNO_2$ and then they were immersed about 20um into this solution and held stationary. At this stage the applied voltage was approximately 2V a.c. and this was found to provide a smooth, pencil-like tip with a diameter of about 1μm.

(b) Insulation

A number of commercially available resins were used but only Epoxylite 6001 "Electrode Insulator" and Inslx-E33 were found to give reasonably consistent results. It was important to clean the etched pieces of tungsten wire in water, absolute alcohol, and xylene before commencing the insulating step. The cleaned electrodes were dipped,

tip down into the Epoxylite resin and slowly withdrawn. They were then quickly inverted and placed in an oven maintained at 250°C, and the resin was cured for 30 minutes. During this stage the resin shrinks and exposes part of the tip, thereby making extracellular recording possible. This coating procedure was repeated three more times before a sufficient degree of insulation was reached. With the Inslx-33 resin the procedure was virtually the same but the curing cycle required only a 2 hour period at room temperature followed by a 12 hour period at 60°C (Green, J.D., 1958).

(c) Testing

Both these insulation procedures produced coated microelectrodes having impedences in the range 3-15M Ohms measured at a frequency of 1 KHz. The method used for testing the electrode resistance was that described by Brown, 1973. This technique relies on generating a constant current signal that produces a voltage across the micro-electrode proportional to the electrode resistance.

Before using the electrodes in an experiment they were also checked for possible leaks in the insulation. Course testing was carried out by watching the appearance of air bubbles on the electrode when a 6v D.C. (Negative) current was passed through the electrode when it was immersed in physiological saline. If bubbling was observed in any region other than the tip of the electrode it was discarded.

3. Stereotaxic and Electrophysiological Recording Techniques

As shown in Fig.1 the pars compacta region of the substantia

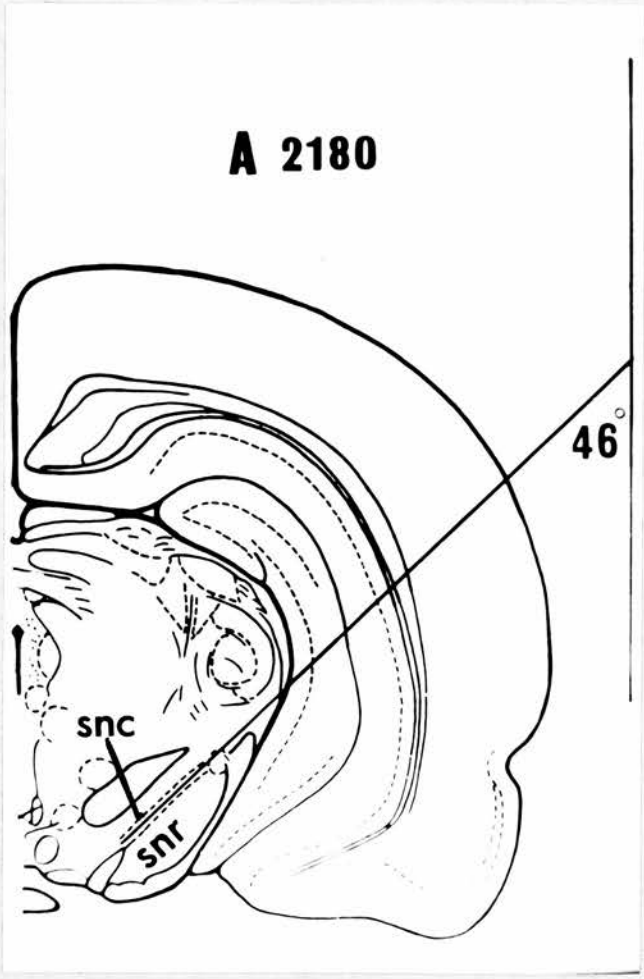

Fig.1

Diagram showing the electrode penetration angle used during the recording of single cells in the zona compacta region of the substantia nigra. Section is from the atlas of König and Klippel, 1963.

Abbreviations: snc, substantia nigra; zona compacta region; snr, substantia nigra, zona reticulata region.

nigra is a narrow layer of cells and the long axis of this region is approximately 46° from the vertical axis of the brain. To enable more pars compacta cells to be recorded in a given penetration, the electrode was introduced at this angle so that it would penetrate along the compacta region.

Male Wistar rats (n=57) weighing approximately 200 grams in weight were anaesthetised with a 3% halothane/air mixture, regulated by a Vapor Halothan vapouriser. Prior to stereotaxic procedures a tracheal canula was routinely inserted and the neck wound closed. The animal was then fixed in a David Kopf stereotaxic frame using blunt ear bars with a 45° tip, usually recommended for guinea pigs. The tooth bar of the frame was fixed at -2.4mm below the ear bar zero. At this stage the animal was maintained under anaesthesia with a 1.5% Halothane/air mixture with a flow rate of 500mls/min. The temperature of the animal was maintained between 36-38°C by a thermostatically controlled heating blanket.

The skull was opened by a midline incision and the skin flaps were pulled to the side. On the subsequent recording side the temporalis muscle was pushed away from the underlying skull. In all preparations bregma was used as the stereotaxic reference point and in order to see this suture clearly, the overlying periosteum was scraped away. Zona compacta cells were located, most reliably using the following coordinates:

anterior posterior	(AP)	-4.5mm
lateral	(L)	7.0mm
vertical	(V)	6.0 - 8.0mm (measured from the overlying cortical surface)

Using the AP and L coordinates it was possible to determine the position of the burr hole in the skull. The burr hole was shaped like a key-hole to enable the lateral position of the electrode to be varied. Following drilling, the clotted blood and dural membrane were removed to prevent any damage occuring to the electrode on entry. The exposed cortex was bathed in warm liquid paraffin to prevent drying of the tissue.

Tungsten microelectrodes were held in a specially adapted David Kopf electrode holder and introduced through the burr hole under microscopic guidance. A small stepping motor (Forth Instruments) was fitted into the holder and by using a microdrive it was possible to advance the electrode by 1μm steps. During recording of cellular potentials the Halothane concentration was maintained at 0.7%.

The microelectrode was attached to a field-effect transistor (2N3819 Radiospares) and electrode potentials were recorded using a Tektronix 5A22N differential amplifier. In each case the indifferent electrode was a silver wire inserted into muscle tissue in the neck region. Sometimes a cotton wool swab, soaked in saline was pushed under the skin and the silver wire was embedded in it. This allowed for better electrical contact. Signals were displayed on a Tektronix D13 dual beam oscillscope. Virtually all 50Hz mains noise and radio interference could be eliminated by suitable filtering. (High frequency -3dB at 3KHz; low frequency -3dB at 1KHz.) Subsequent to display on the oscilloscope the cellular records were analysed using a Digitimer D-130 Spike Processor, in combination with a Biomac 1000.

Initially the microelectrode was advanced through the cerebral cortex until neurones could be isolated and held for periods of up to ten minutes. This gave a good indication of the recording ability of a given electrode. At the end of each experiment the recording sites were marked by passing a current of a few microamps D.C. through the electrode until approximately 100-200 microCoulombs of charge had been passed. This produced a lesion that was "pear-shaped" and usually 50-100 microns in diameter.

4. Physiological Monitoring of Blood Pressure, Electrocardiogram (EKG) and Respiratory Rate

(a) Blood Pressure and ECG.

In some experiments in which drugs were administered intravenously the above three physiological parameters were measured. (number = 18)

The blood pressure of the animals was measured by means of an intravenous cannula (Portex cannula 2 FG) inserted into the femoral artery. Prior to insertion the cannula was flushed through with a 5000 U/ml. Heparin solution and subsequently flushed out with one or two rinses of physiological saline. This resulted in a cannula which seldom blocked during an experiment. The cannula was fitted to a pressure transducer (Statham) and the resulting electrical output was passed to a Grass "Polygraph" D.C. amplifier and the signal was plotted with a pen recorder. Before each experiment the pen recorder was calibrated using a small mercury manometer.

A three point EKG was obtained by measuring the potential difference across the animals chest, using two I9 G needles as

electrodes. A needle inserted into the leg acted as an indifferent. The output voltages were amplified by a Grass "polygraph" high gain A.C. pre-amplified and recorded on chart paper.

(b) Respiratory Rate

Respiratory rate was recorded simultaneously with the previous two parameters. This was measured, quite simply, by connecting an arm of the Y-piece tracheal cannula to a Grass volumetric pressure transducer. Again the output signal was amplified and displayed on the pen recorder.

5. Intravenous Cannulation

Administration of drugs was usually via the intravenous route. In these preparations the femoral vein was cannulated with a Portex nylon intravenous cannula 3FG. To prevent recurrent problems due to blockage of the cannula, physiological saline was infused through it at a constant rate of 0.016 ml./min. by a Watson-Marlowe perfusion pump. When drug injections were made through the rubber cap of the cannula the infusion rate of saline was stepped up to 0.032 ml./min. for 30 seconds in order to wash in the drug.

6. Routine Histological Staining Procedure: A modified Kluver-Barrera Technique

The staining technique used was a slightly modified version of the one first reported by Klüver and Barrera, 1953.

At the end of all acute experiments the animal was deeply anaesthetised with 3% Halothane and decapitated. The brain was removed

and a 4mm section of tissue, containing the entire SN was dissected out. This was mounted on a cryostat chuck and frozen by CO_2 expansion. Sections were cut at 15um thickness and every third one was collected in the region of the electrode tract. The sections were mounted on coverslips, in preparation for staining.

The sections were treated as follows:

(a) Washed in 95% Ethanol for 5 mins.

(b) Stained for 10 mins. in a filtered 0.1% Luxol Fast Blue solution containing 5ml. of 10% acetic acid in every 1000ml. of solution and kept at a temperature of 40°C.

(c) Washed in 95% Ethanol to remove excess stain.

(d) Washed in distilled water.

(e) Differentiated by brief immersion (10 secs.) in 0.05% lithium carbonate solution.

(f) Differentiation continued in 70% Ethanol for 30 secs.

(g) Washed in distilled water.

Steps e-g were repeated until only the white matter in the section was stained light blue against a clear background.

(h) Washed thoroughly in distilled water.

(i) Stained for 10 mins. in warm (50°C.) cresyl violet solution (0.2%) containing 5 drops of 10% acetic acid to every 30mls. of solution.

(j) Differentiated in several changes of 95% Ethanol.

(k) Dehydrated in absolute Ethanol, cleared in xylene and mounted in Canada balsam or D.P.X. ready for microscopic examination.

RESULTS

1. Histological Localisation of DA neurones in the substantia nigra

The DA containing cell bodies in the substantia nigra and ventral tegmental area VMT were localised and mapped out using the Falck-Hillarp histological technique. Serial sections containing the DA neurones were carefully examined under dark field illumination and the DA cell distribution at various planes of section was photomicrographed. Fig.2(a) and (b) are photomontages of the distribution of the cell groups A9 and A10 superimposed on the corresponding plane of section taken from the atlas of König and Klippel, 1963.

In Fig.2(a) the plane of section is at the rostral region of the substantia nigra. It shows that the DA cells are localised predominantly in the zona compacta region, in the A9 group of cells (Dahlström and Fuxe, 1964(a)). These cells are small to medium in size (15-25 microns), oval or round shaped and grouped quite closely together in a layer 5-6 cells thick. The cell group A10 was also present at this level and the cells were identical to those found in A9. There is no clear boundary between these two cell groups and they were viewed as being continuous with one another. In the electrophysiological data presented later in this section this was the plane of section at which the majority of DA cell were recorded.

Fig.2(b) is a composite diagram taken at a more caudal plane of section and it is apparent that the DA cell distribution differs from that shown in the previous figure. The majority of the DA cells are grouped together medially and completely overlie the interpeduncular

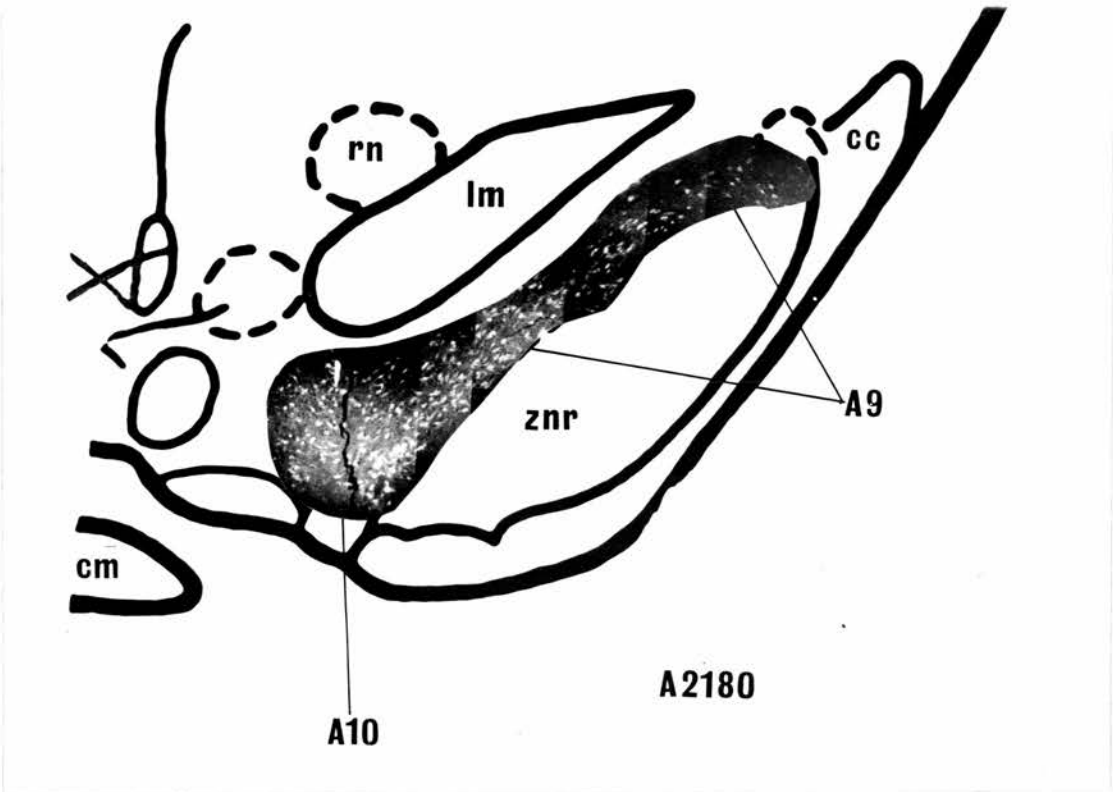

FIGURE 2(a)

Composite of individual photomicrographs taken throughout the ZC and VMT region of the anterior midbrain. The tissue was treated according to the method of Falck and Hillarp 1964. The photomontage shows the location of the fluorescent DA cells in A9 and A10 with respect to other brain structures found at this plane of section A2180 taken from the atlas of König and Klippel.

cm, corpus mammillare; znr, zona reticulata; rn, red nucleus; lm, lemniscus medialis; cc, crus cerebri.

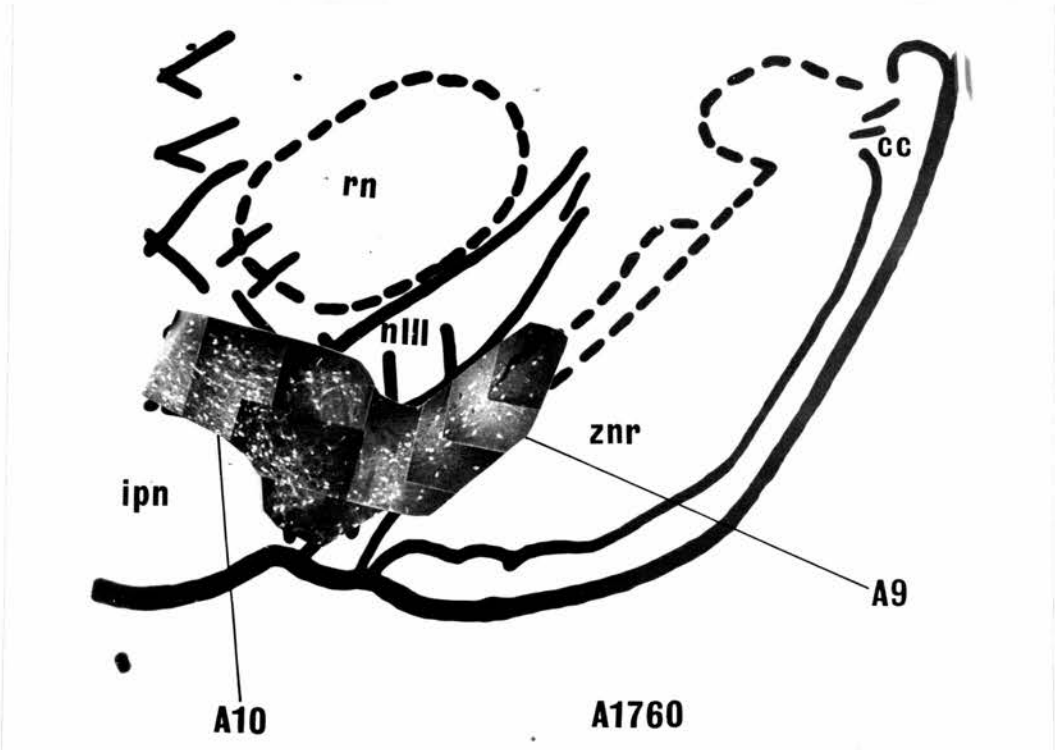

FIGURE 2(b)

Location of DA neurones in the caudal substantia nigra and the VMT region. This figure was constructed in a similar manner to Fig. 2(a) but it should be noted that it does not show the complete lateral extension of the A9 region. The plane of section is A1760 taken from the atlas of König and Klippel 1970.

nIII, oculomotor nerve; ipn, interpeduncular nucleus.

nucleus (IPN). This group is the A10 region. Also shown in this figure is part of the A9 group which extends laterally along the zona compacta region of the substantia nigra. At this plane of section A9 and A10 cell groups are more or less continuous with each other.

A third DA cell group A8 was mapped out but it is not illustrated here since no extracellular records were collected from that region. This group is more diffuse than the other two groups and is situated dorso lateral to the substantia nigra and extends medially, dorsal to the medial lemniscus.

In all the planes of section studied very few DA cell bodies were found to be present in the zona reticulata region of the SN.

2. Electrophysiological and pharmacological identification of DA-containing neurones in the substantia nigra and ventromedial tegmental area.

(a) Electrophysiological characteristics of SN and VTA neurones

The initial electrophysiological recordings from the SN and VTA confirmed that two groups of neurones were present. Both groups were spontaneously active but differed in their electrical characteristics as well as their anatomical location. All the cells recorded in the zona compacta and VTA area in this initial study (number = 17) had firing frequencies in the range 1-14 spikes/second (Hz.), whereas the cells located in the zona reticulata region (number = 16) invariably fired much faster, in the frequency range 18-85Hz. Figure 3 is a

summary diagram of 32 representative recording sites in the region of the SN and VTA. The firing rate of the neurones in the zona compacta and VTA was 7.9 ± 3.6 (mean$\pm$S.D.) and was significantly different from the zona reticulata neurone firing rate of 36 ± 15.3Hz. ($p < 0.001$; Students t-test 2-tailed).

Initially, if these faster zona reticulata cells were recorded during an electrode penetration then further step-wise penetrations along a line located more dorsal usually resulted in records being obtained from the slower cells. Dorsal to the zona compacta it was possible to record axonal potentials from fibres of the medial lemniscus. These potentials had a short spike duration (1ms) and were very easily damaged if the electrode was moved as little as 5 microns. These units also showed a strong tendency to fire with a burst-like activity which was unlike that of other cells recorded in the region. Medially the fibres of the third cranial nerve, the oculomotor, could be recorded, but again these potentials could be distinguished from somatic spikes by their short spike duration (1ms) and extreme fragility.

The zona reticulata cell spikes were typically tripasic in shape and had a duration of 2-2.5ms. Figure 4 illustrates an example of this type of cell. A typical firing pattern of a zona reticulata cell firing at 25Hz. is also shown in Figure 4. These cells fired very regularly with no tendency to fire in bursts.

In contrast to the zona reticulata cells, the zona compacta and

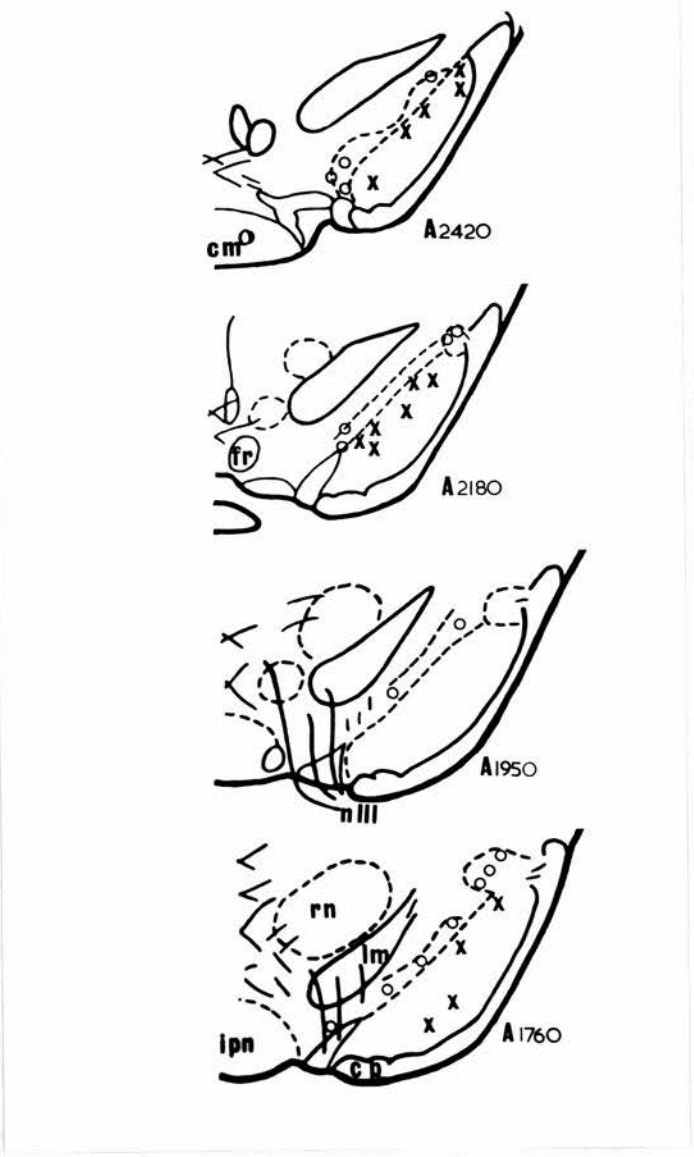

FIGURE 3

Schematic diagram of electrode recording sites at four frontal planes through the region of the anterior midbrain classifying the cell firing rates into two distinct firing frequency ranges. The empty circles (O) denote cells that fired in the range 1-14 spikes/sec., with a mean firing rate of 7.9 $\pm$ 3.6 spikes/sec. (mean $\pm$ S.D.). The crosses (X) denote cells which fired in the frequency range 18-85 spikes/sec., with a mean firing rate of 36 $\pm$ 15.3 spikes/sec. The slower firing group of cells also had a longer spike duration than the faster firing group and showed a tendency to fire with a slight burst-like activity.

The diagram is modified from the stereotaxic atlas of König & Klippel 1970

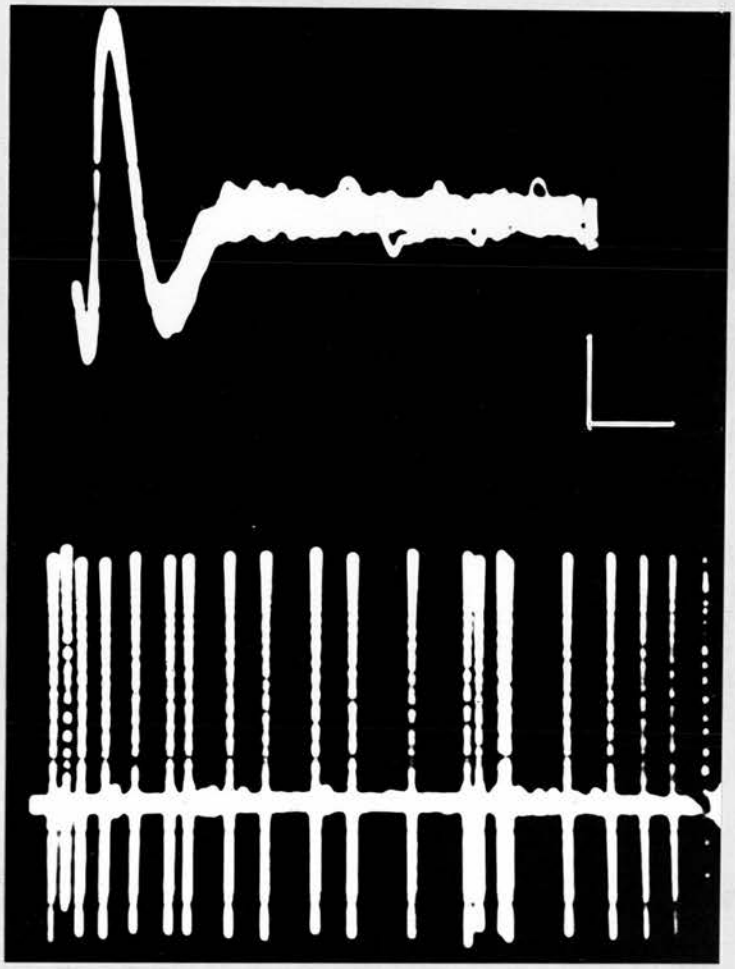

FIGURE 4

Oscilloscope traces of the extracellular potential (upper trace) and firing pattern (lower trace) of a neurone located in the zona reticulata region of the substantia nigra. The rat was anaesthetised with a 0.7% Halothane/air mixture. Both traces were recorded at the same amplification. The sweep time of the lower trace is 1 second and the calibration marks are 1ms. (horizontal) and 0.1 mV a.c. (vertical).

VTA cells showed a slight tendency to fire in a burst-like activity, 2-3 spikes in a burst. A typical firing pattern trace is shown in Figure 5. It was always observed that the spike amplitude decreased during a burst of spikes, a finding also reported by Bunney et al., in 1973. A very noticeable characteristic of the DA cells was the long duration of the action potential, which was in the range 2.5-3.5ms. A typical extracellular potential of a cell located in A9 is shown in Figure 6. This also illustrates that the action potentials were always complex in nature. This was a very consistent finding and was the best electrophysiological criterion for identification of DA cells. The extracellular records were very stable, in many cases it was possible to move the electrode 20 microns in either direction without losing the cell or changing the spike shape or amplitude. Cells having a zona reticulata firing pattern were never found to have a long spike duration, another distinguishing characteristic between these two cell groups.

In three experiments the cell recording site was lesioned in the normal manner and the tissue was treated according to the Falck-Hillarp technique. Such a lesion site is shown in Figure 7. It was found to be rather difficult to locate these lesion sites, due to the number of freezing fractures in the tissue. The lesion site shown was located in the dorsal part of A10 and in the region of a cell which had all the electrophysiological characteristics of a presumed DA cell and which was also inhibited by administration of apomorphine hydrochloride, given intravenously.

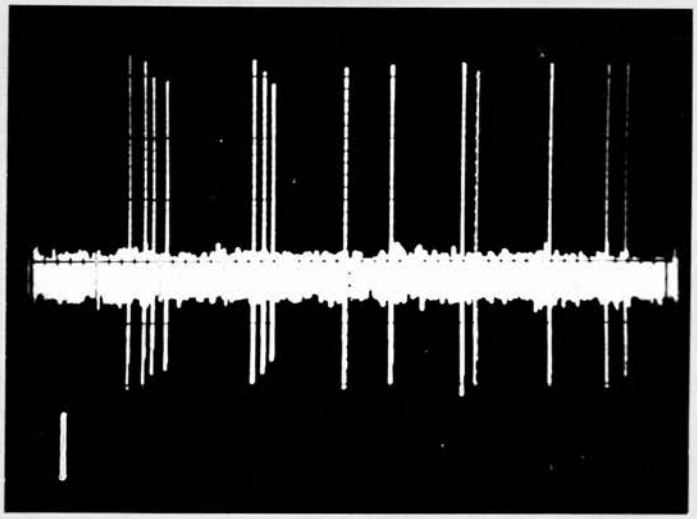

FIGURE 5

Oscilloscope trace of typical firing pattern of a neurone located in the ventral tegmental area. Note the characteristic bursts of spikes with a progressively decreasing amplitude.

This is a complete oscilloscope trace taken with the sweep time set on 2 seconds.

The vertical calibration mark represents 0.1 mV.

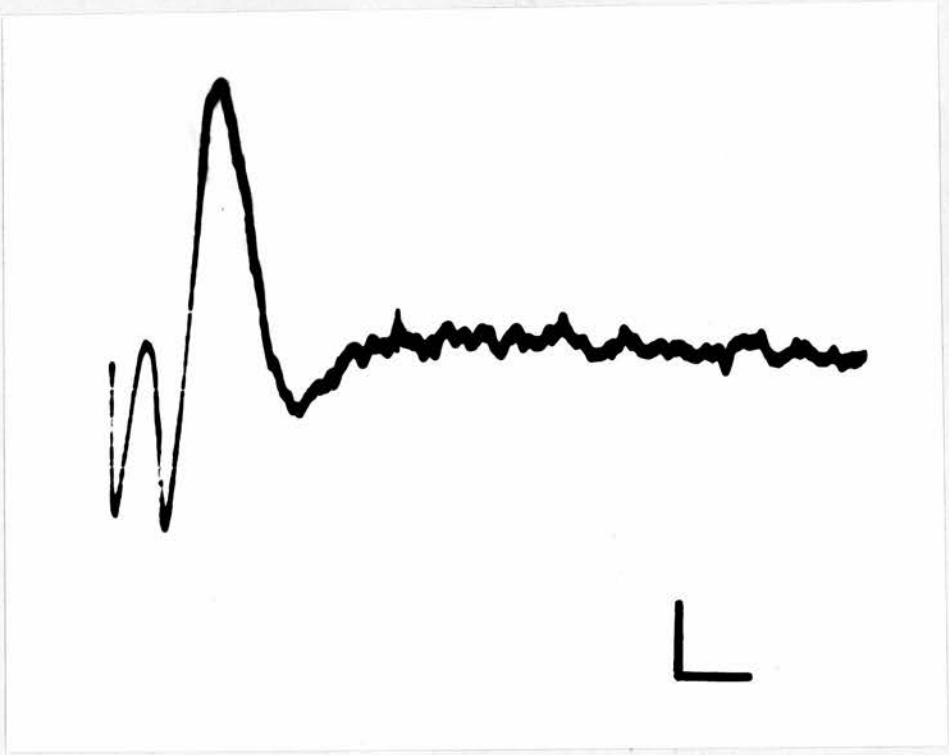

FIGURE 6

Typical oscilloscope trace of the extracellular potential recorded from a neurone in the zona compacta region of the substantia nigra. The preparation was anaesthetised with a 0.7% Halothane/air mixture. The calibration marks are 1 ms (horizontal) and 0.1 mV (vertical).

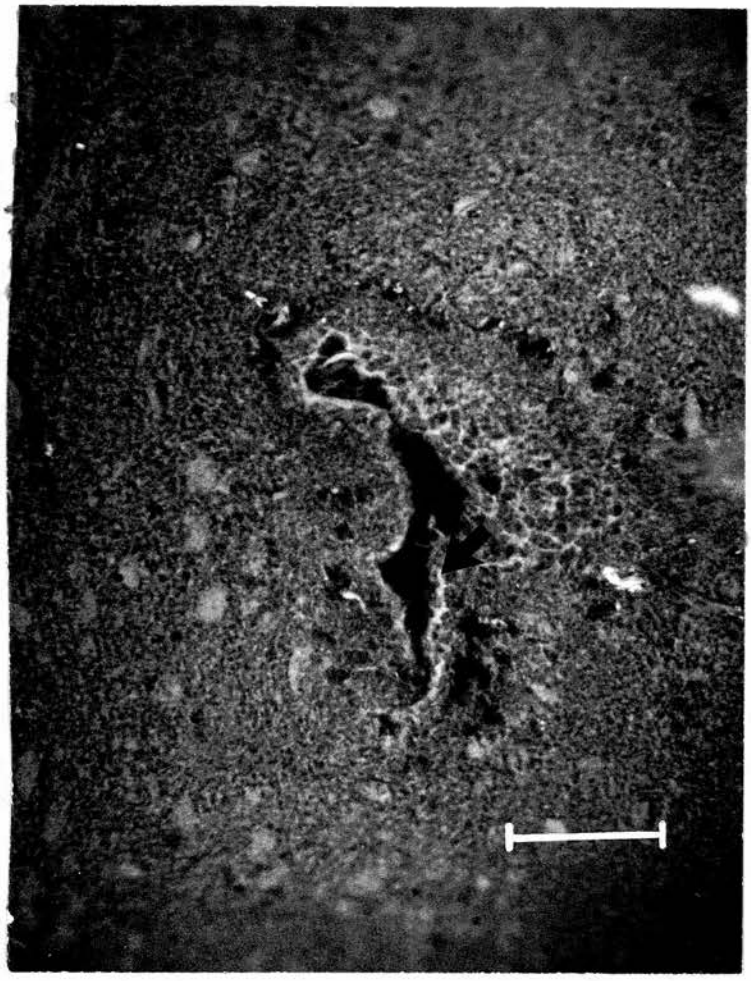

Figure 7

Electrolytic lesion (arrow) in the region of DA-containing cells (A10) in the substantia nigra. This lesion was produced by the passage of 200uCoul. of charge through a tungsten microelectrode, following the extracellular recording of a single unit with the electrophysiological characteristics of a presumed DA-containing neurone. The tissue was treated according to the Falck-Hillarp technique but the DA fluorescence was relatively weak due to a fault in the freeze-drier which did not permit the development of sufficient vacuum during the initial freeze-drying procedure.

Scale bar: 100um

2. (b) The Effects of the administration of DA agonist and antagonist drugs on the firing rates of neurones located in the SN and VTA

Twenty animals were prepared for intravenous administration and in 18 of these preparations blood pressure and respiration were continuously monitored. Sixteen cells were located and held for periods greater than 2 hours. In each experiment only 1 cell was studied. Of these cells 10 had the characteristics of presumed DA cells as previously outlined, and 6 were from sites outwith the ZC and VTA with electrical properties very different from DA cells. Figure 8 is a summary diagram of the recording sites in all the experiments. The non-DA like cells are denoted by (X) and the DA cells by empty or filled circles.

Following the location of a cell, a control period of at least 30 minutes was recorded and drugs were subsequently administered only if the cell had a stable firing rate. Apomorphine hydrochloride 0.1 mg./kg. was then injected and it was found that all presumed DA cells were strongly or totally inhibited by this dose. In the firing rate histogram shown in Figure 9, apomorphine completely inhibited a DA cell located in A9. This cell had a control firing frequency of 6Hz. This inhibition occurred within 20 secs. following drug administration and the cell showed a rapid recovery to about 30% of its basal rate within 3 minutes. Thereafter, the cell firing rate slowly increased and had recovered its control value within 40 minutes of the apomorphine administration. Also shown on this figure is the blood pressure record accompanying the DA cell firing changes. This fell very rapidly and reached a nadir of 55mm Hg within 40 secs. There was a marked decrease in pulse pressure as the blood pressure

dropped. However, there was a rapid return of the mean blood pressure to pre-injection levels within 5 minutes. The pulse pressure slowly increased and after 20 minutes it had attained a normal level.

All 10 cells with presumed DA characteristics and located in the region of the DA containing cells shown in Figure 8 responded in a similar manner, except that 4 cells were not totally inhibited by this dose of apomorphine. In 2 experiments 2 doses of apomorphine were given at 10 minute intervals. Figure 10 is a firing rate histogram of a DA cell when treated in such a way. In this case the cell was not completely inhibited but was depressed to about 15% of its control firing rate. A second identical dose of apomorphine, given at a time when the cell had recovered to 50% of its control rate, decreased the firing rate to the same level as the first injection.

It was a consistent finding that DA cell spike amplitude markedly increased in response to 0.1 mg./kg. apomorphine. It seemed likely from the initial few cells studied that this amplitude change was correlated with the change in firing frequency of the cell. During the course of a DA cell record following apomorphine 20 spikes were sampled at timed intervals and from these spikes the mean interval between spikes and the mean amplitude were calculated. The data for one such experiment are shown in Figure 11. The spike amplitude was found to be highly correlated with the mean spike interval ($r = 0.94$). All DA neurones studied showed an almost identical response to this dose of apomorphine.

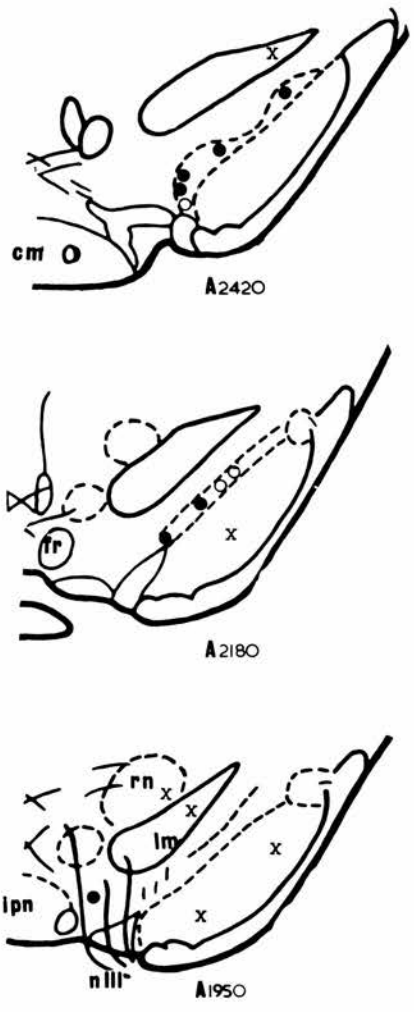

FIGURE 8

Schematic diagram of electrode recording sites at which neurones were tested for their responsiveness to intravenously administered apomorphine, amphetamine and haloperidol. The recording sites are projected onto the appropriate transverse section (indicated by number underneath) taken from the atlas of König and Klippel, 1963. Shown are 10 sites at which the firing rate of cells was inhibited by apomorphine, 0.1mg./kg. (●,○). The open circles denote sites at which neuronal activity was increased by haloperidol 1mg./kg. and inhibited by apomorphine. The filled circles in A2180 denote cells which were inhibited by both apomorphine and amphetamine 1mg./kg. Cells denoted (X) failed to respond to apomorphine.

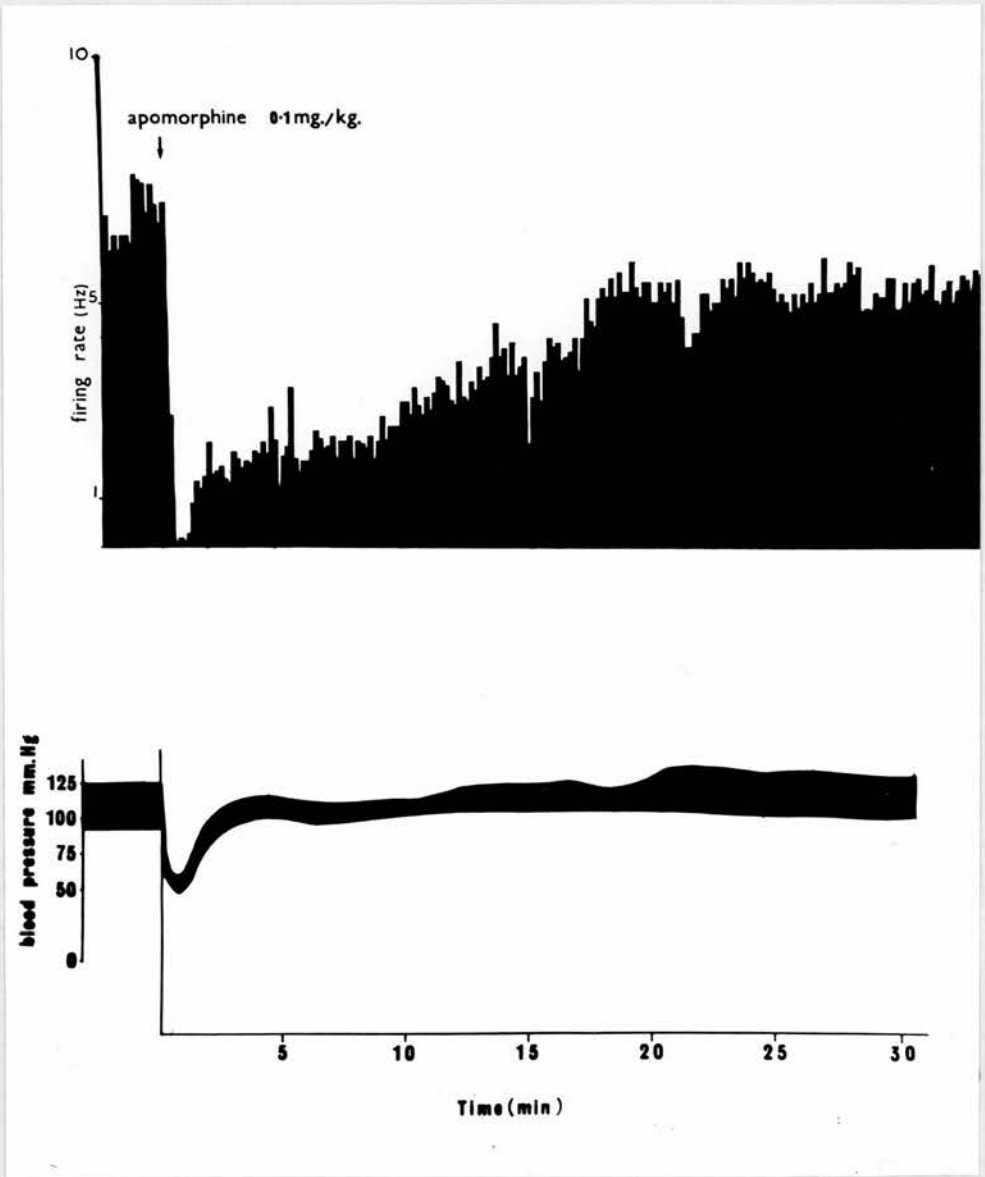

FIGURE 9

Inhibition of DA neurone firing rate by apomorphine and the accompanying effects on blood pressure. The upper trace illustrates the effect of intravenous administration of apomorphine hydrochloride 0.1 mg./kg. on the firing rate of a neurone located in the A9 region of the SN. The firing rate was calculated from consecutive 10 second spike counts. The simultaneous blood pressure changes shown in the lower trace are temporally aligned with the upper trace. Note that the longer vertical axis of the lower trace corresponds to the time of apomorphine administration, shown in the upper trace.

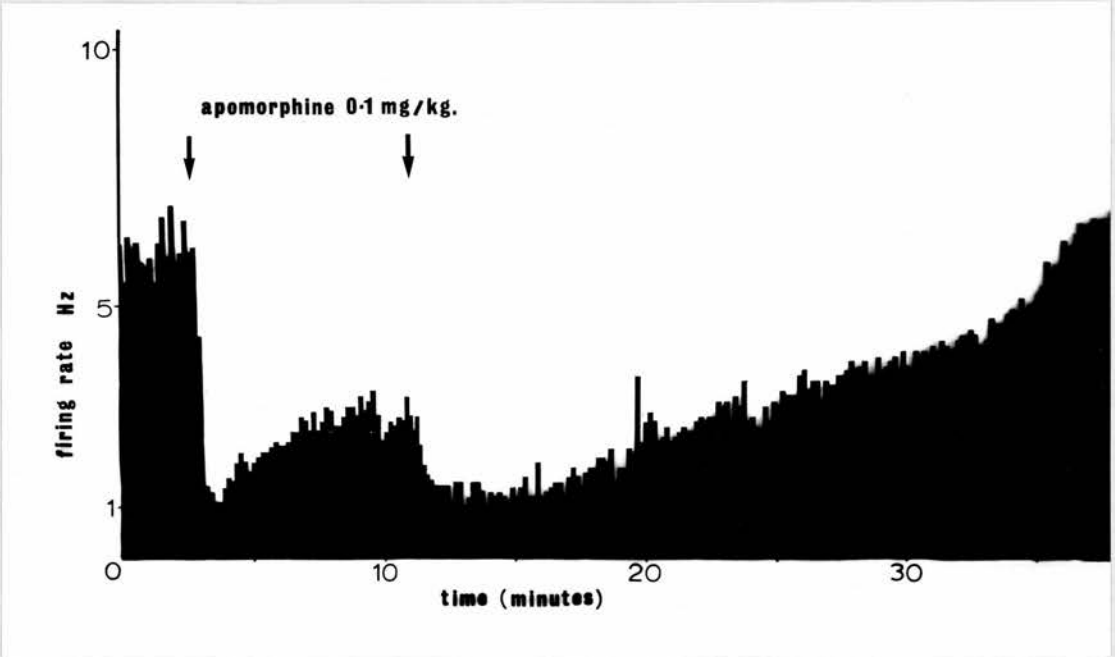

FIGURE 10

Response of a neurone located in the zona compacta region of the SN to two doses of apomorphine hydrochloride administered intravenously. The doses of apomorphine, 0.1 mg./kg. in each case, were administered at the times indicated by the arrows. The firing rate was calculated from consecutive 10 second spike counts.

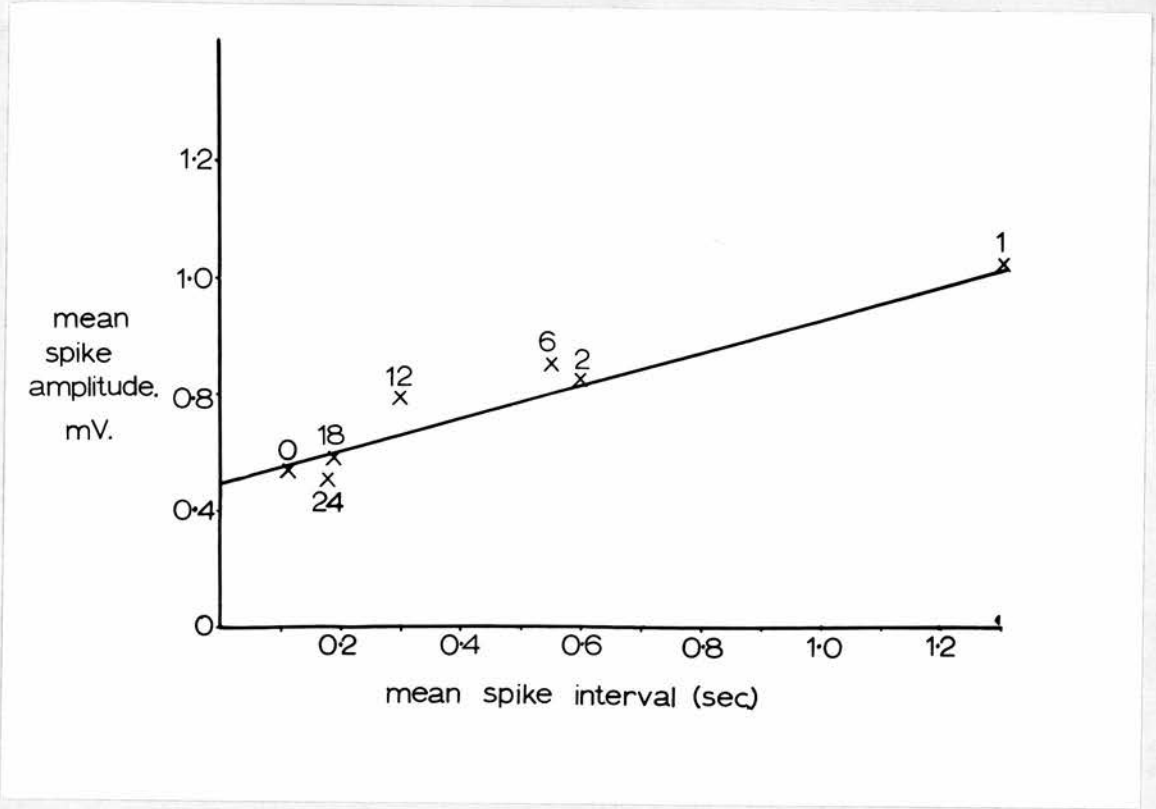

FIGURE 11

Regression line showing the correlation between DA cell amplitude and mean firing interval following intravenous administration of apomorphine. The mean firing interval and amplitude were calculated from samples of 20 spikes photographed at various timed intervals throughout the cell record. The number adjacent to each point represents the time in minutes after apomorphine administration (0.1 mg./kg.) at which the 20 spike samples were collected. Time (0) refers to the control values measured before apomorphine administration.

Single units located in the red nucleus, medial lemniscus and zona reticulata did not change their firing rate significantly in response to 0.1 mg./kg. apomorphine. Figure 12 shows the effect of this dose of apomorphine on the firing rates of cells located in the red nucleus and zona reticulata. The red nucleus cell did not respond but the zona reticulata cell appeared to show a greater variation in its firing frequency, and if anything it showed a tendency to increase its firing rate. There was also no change in the spike amplitude of these cells following administration of this drug

The initial pharmacological studies on DA neurones, by Bunney et al., 1973, showed that d-amphetamine inhibited these neurones. In the present experiments this drug was used in only 2 instances because of the long duration of the drug effect and the difficulty in holding the cell until control firing was again reached. The results of 1 d-amphetamine experiment are shown in Figure 13. This cell was initially inhibited by apomorphine 0.1 mg./kg. after a 90 minute interval, when the firing rate had returned to a steady level, d-amphetamine 1mg./kg. was administered. The DA cell was inhibited to about 30% of its control firing rate within 40 secs. and had recovered to 50% within 5 minutes. In this experiment the cell amplitude was found to increase, both in response to apomorphine and amphetamine.

The neuroleptic drugs chlorpromazine and haloperidol were found to increase DA unit activity. 3 DA cells were tested, firstly with apomorphine which caused an inhibition in all three cells (see Figure 8) and secondly with haloperidol, which was found to cause a

slight increase in firing rate when given in a dosage of 0.1 mg./kg. Figure 14 illustrates the blocking effect of such a dose of haloperidol on the depressant action of apomorphine on the DA neurone. In addition to causing a slight increase in DA cell firing rate haloperidol decreased the spike amplitude and the firing pattern was characterised by repeated bursts of spikes with progressively decreasing amplitudes as shown in Figure 15. This made it rather difficult to set the window levels of the spike analyser so that all the spikes could be counted without background interference. Haloperidol was found to have a very long lasting effect, greater than 2 hours and it was impossible to hold a cell until it had recovered to its basal firing rate. Figure 16 shows the effect of 0.1 mg./kg. haloperidol on blood pressure and respiration. There was a very rapid fall in blood pressure within 20 secs., very similar to the effect of apomorphine and it had recovered to a normal level within 3 minutes.

Figure 17 shows the effect of chlorpromazine, administered intraperitoneally, on a neurone located in the A10 region. This neurone was not included in the summary diagram shown in Figure 8. In this instance the DA cell firing rate more than doubled in response to a small dose of chlorpromazine. This was the only cell which was studied following administration of this neuroleptic.

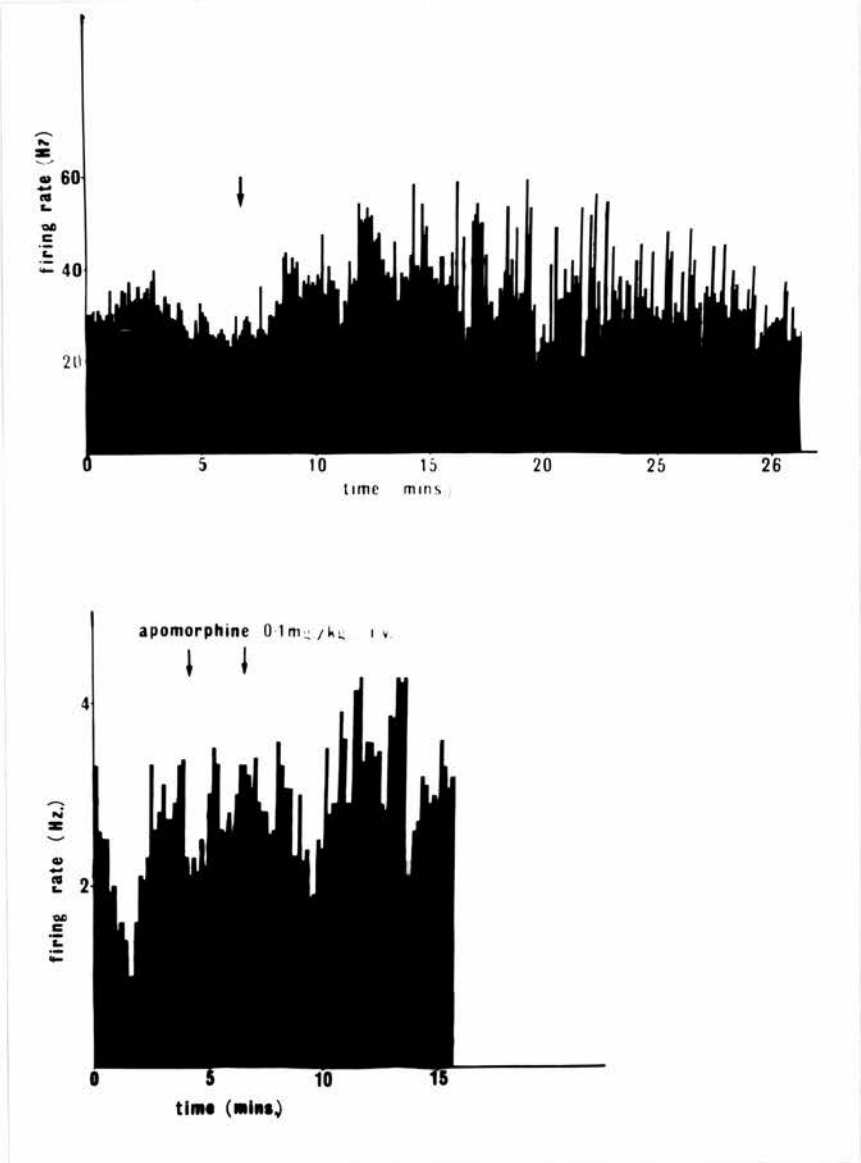

FIGURE 12

The effect of apomorphine (0.1 mg./kg.) on the firing rate of a neurone located in the zona reticulata (upper) and red nucleus (lower). The firing rates were calculated from consecutive 5 second spike counts (zona reticulata cell) and 10 second spike counts (red nucleus cell). The arrow in the upper histogram indicates the time of apomorphine administration. Neither neurone was inhibited by this dose of apomorphine.

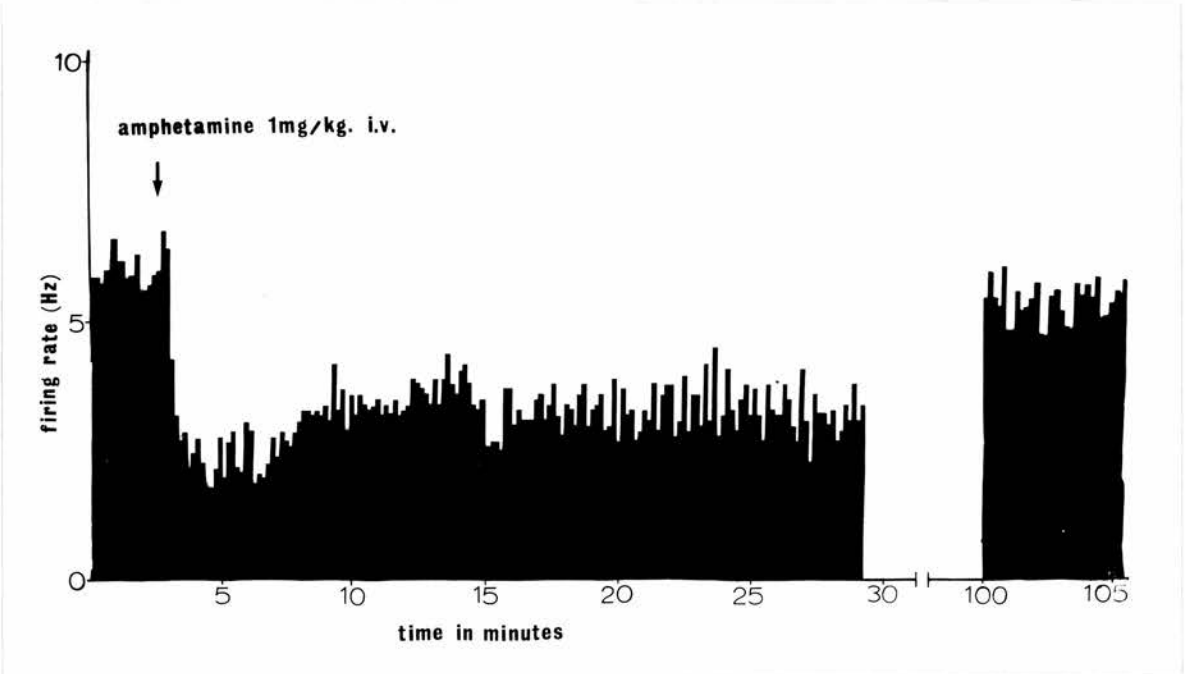

FIGURE 13

Effect of D-amphetamine 1.0 mg./kg. intravenous on the firing rate of a typical cell located in the zona compacta region of the substantia nigra. This drug was found to cause a long-lasting depression of firing rate and the cell recovered 100% control values within two hours. This cell was initially depressed by an intravenous dose of 0.1 mg./kg. apomorphine and allowed to recover control firing rate before administration of d-amphetamine. The firing rate was calculated from consecutive 10 second spike counts.

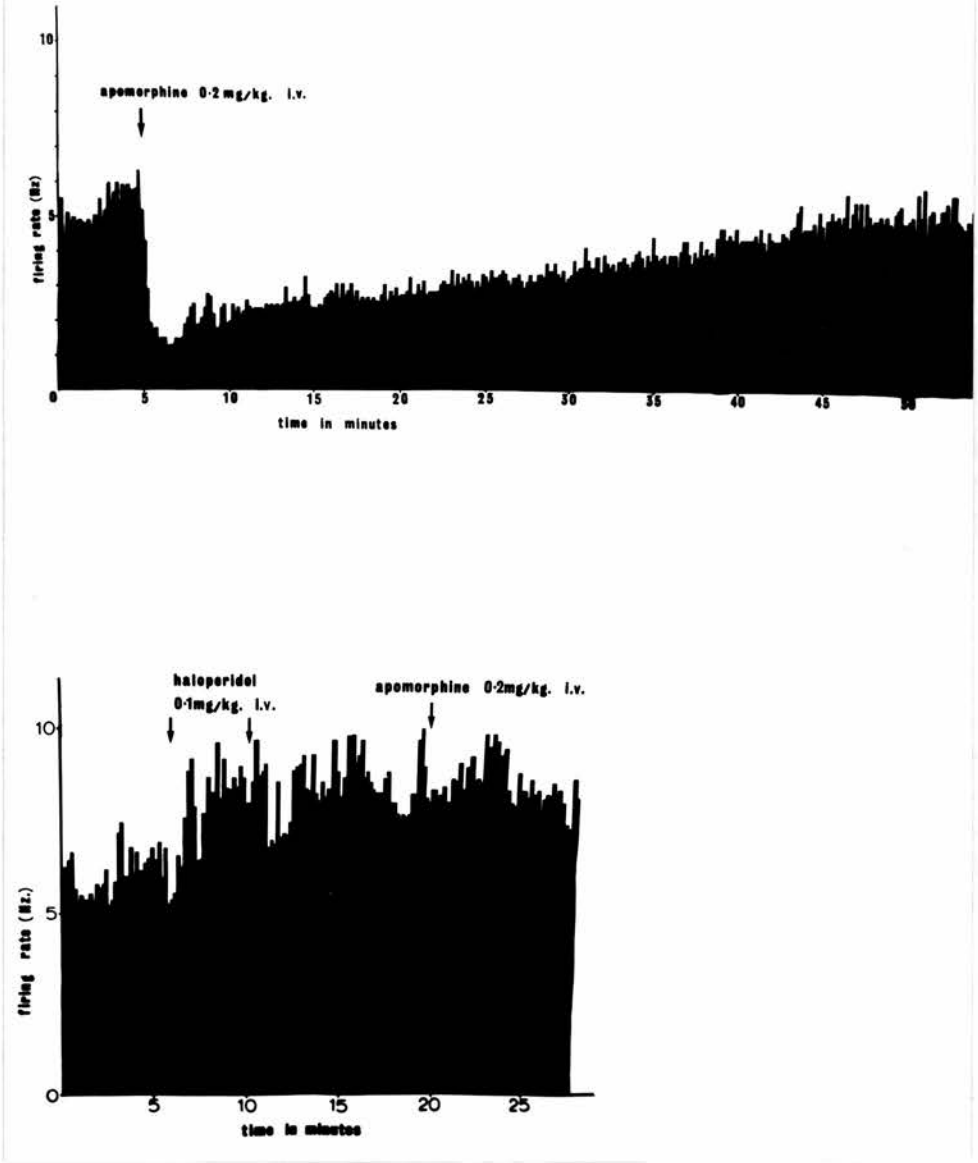

FIGURE 14

Blocking effect of haloperidol on the depressant response of a zona compacta neurone to apomorphine. The upper and lower traces are a continuous record but the axes of the lower histogram are different from the upper. The neurone was strongly inhibited by the intravenous administration of 0.2 mg./kg. apomorphine hydrochloride (upper trace). The cell recovered normal firing rate and haloperidol was administered as indicated by the arrow and in the dosage shown. The firing rate of the neurone increased slightly and a subsequent dose of haloperidol was found to have no effect on the firing rate. The effect of a second dose of apomorphine was blocked by administering haloperidol (N = 3).
In both traces the firing rate was calculated from consecutive 10 second spike counts.

FIGURE 15

Oscilloscope trace showing the firing pattern of a neurone located in the zona compacta region of SN following the administration of haloperidol 0.1 mg./kg. intravenously. The neurone had a marked tendency to fire in a burst-like manner with spike amplitude progressively decreasing within the bursts.

The calibration marks are 10 ms. (horizontal) and 0.2 mV (vertical).

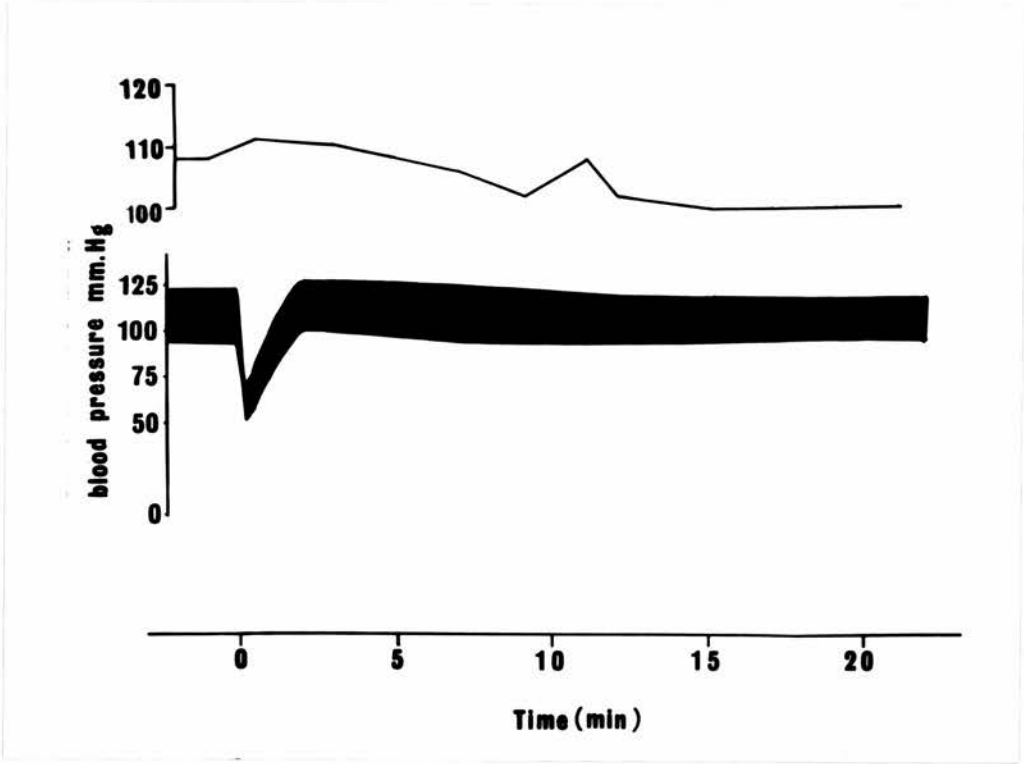

FIGURE 16

The effect of haloperidol 0.1 mg./kg. given intravenously, on the respiration rate and blood pressure of the halothane anaesthetised rat. Note that both traces are temporally aligned and the drug injection was at time zero. The unlabelled ordinate shown in the upper trace is rate of respiration per minute.

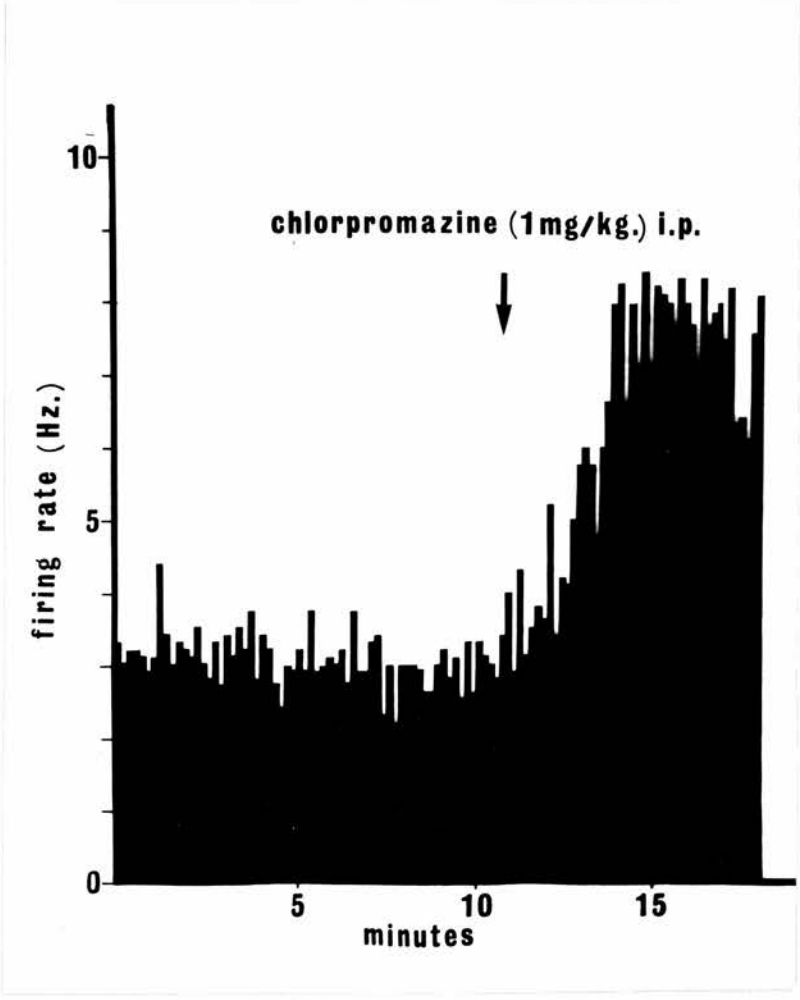

FIGURE 17

Effect of chlorpromazine 1mg./kg. given intraperitoneally, on the firing rate of a neurone located in the ZC region of the substantia nigra. The arrow denotes the time of drug administration. This cell is not included in the recording sites summarised in Fig.8.
Initially this cell was strongly depressed by apomorphine 0.1 mg./kg., given intravenously and the cell recovered normal firing rate within 1 hour.
The firing rate was calculated from consecutive 10 second spike counts.

DISCUSSION

Extracellular unit recording from histologically and neurophysiologically identified DA neurones have been reported by three other groups of workers (Bunney et al., 1973(a), (b); Rebec, Groves et al., 1975; Dray, Gonye and Oakley, 1976). The most detailed description of the electrical characteristics of the neurones in the zona compacta and ventral tegmental areas was reported by Bunney et al., 1973(a) from gallamine paralysed, as well as chloral hydrate and halothane anaesthetised preparations. In this present section of work the zona compacta and VTA DA cells have been electrophysiologically identified according to criteria identical to those applied by Bunney and his co-workers i.e. firing rate, spike duration and firing pattern.

The firing rate of zona compacta and VTA neurones was clearly slower than the zona reticulata neurones and a clear distinction could be drawn between these cell groups on this criterion alone. Bunney reported that the zona reticulata cells were firing at a "much faster rate and had no bursting activity" when comparing them with the zona compacta and VTA neurones. This finding was confirmed in the present studies. However, zona reticulata cells firing at a rate less than 20 Hz could sometimes be located and in these instances it was possible to classify the cells further according to spike duration and firing pattern criteria. The majority of presumed DA cells recorded in the present experiments were located in the zona compacta region and the firing rate of these cells was in the same

frequency range as cells recorded in the VTA region.

The angular stereotaxic approach of the electrode proved to be quite useful and ensured that between one and ten DA neurones could be recorded in one penetration. A major problem encountered was in the accurate estimation of the lateral coordinate, but throughout the course of the experiments the firing characteristics of adjacent cell populations became recognisable. Dorsal to the zona compacta it was always possible to record from the axons of the medial lemniscus and this was useful in estimating the dorsal position of the electrode as well as aiding the accurate anterior-posterior position. Another useful electrophysiological signpost was the third cranial nerve fibres coursing ventrally through the medio-caudal region of the SN. Therefore, it was usually possible to locate substantia nigra neurones within two or three electrode penetrations.

All presumed DA cells located in the ZC and VTA fired in the frequency range 2-14Hz with the majority firing in the range 2-10Hz. This was similar to the firing rates reported by Bunney et al., 1973 in chloral hydrate anaesthetised animals. It is apparent from the same group's findings that anaesthesia increases the firing rate of DA cells. In gallamine-paralysed rats the DA cells fired with a frequency of 2-6 Hz without a burst like activity but the same cells, when subjected to chloral hydrate anaesthesia increased their firing rate to 3-9 Hz and assumed a marked burst like activity. Halothane was the only anaesthetic used in the present experiments because the preparations could be maintained at a constant level of anaesthesia, an important consideration when studying a population of cells that

are anaesthetic-sensitive. The DA cells had characteristics similar to those reported in chloral hydrate anaesthetised animals except that the burst-like activity, prevalent under these conditions, was less pronounced. Groves and Rebec 1975 did not report the firing characteristics of the zona compacta cells recorded in their experiments, except that they fired in the range 4-10 Hz. Dray et al., 1976, reported from urethane pentobarbitone-anaesthetised rats that zona compacta neurones fired at a slow firing rate 11.6 $\pm$ 0.9 Hz and "often showed irregular bursts of activity".

Bunney et al., 1973, reported that DA cells had a biphasic spike shape and they fired, under chloral hydrate anaesthesia, with a marked burst-like activity characterised by a progressively decreasing spike amplitude and increasing spike duration. Glass micropipettes were used in these previous studies and this may explain the difference in spike shape from that obtained in the present experiments. It could be, of course, that these authors did not examine the spike shape very closely. Certainly from the experiments reported here the ZC and VTA cells all had a spike duration in the range 2.5 - 3.5ms. and usually had a complex spike shape. The cells recorded in adjacent areas to the substantia nigra did not have these unusual electrical characteristics. In general, apart from differences in spike shape, the presumed DA cells reported in these experiments bear a striking resemblance to those reported by other workers.

As well as the routine Klüver-Barrera histological staining technique, tissue was examined with the Falck-Hillarp fluorescence

method. In a few experiments it was conclusively shown that the electrode recording site was in a DA cell-containing area. Bunney et al., 1973, iontophoresed l-dopa through the glass micropipette at the recording site and thereby enhanced the DA cell body fluorescence, if these cells were in the immediate vicinity. Neither of these techniques conclusively prove that the identified cells were DA containing but they provide strongly supportive evidence.

Hökfelt and Ungerstedt, 1973, reported that there were two distinct cell groups in the zona compacta region of the substantia nigra, based on ultrastructural differences observed in the electron microscope. Type 1 cells were found to have a cytoplasm with a comparatively high density, with a well developed regularly arranged rough endoplasmic reticulum. These cells were thought to be DA-containing. Type II cells had a lighter cytoplasm and a less developed, often irregular rough endoplasmic reticulum. The former group of cells was selectively destroyed by intra-nigral injection of 6-OH DA. Bunney et al., 1973(a), performed similar 6OH DA nigral lesions and reported that no presumed DA cells could be recorded in such preparations. It is highly likely, especially in view of the specificity of these 6OH DA lesions, that the DA cells were specifically destroyed. This provides the best evidence to date that the presumed DA cells are indeed the dopaminergic cells of the substantia nigra. If type II cells were recorded by the micro-electrodes then they must either have identical electrical characteristics to type I cells or they are normally silent since only one population of cells could be recorded from this area.

Another possible electrophysiological test that could have been applied to the DA neurones was antidromic invasion, following caudate nucleus stimulation. This was not attempted in the present experiments for two main reasons. Firstly, all the nigral cells do not project to the caudate nucleus (Ungerstedt, 1971) and thus, antidromic activation would not be possible in A10 cells, which predominantly project to the mesolimbic brain areas. Secondly, there exists the possibility of a non-dopaminergic monosynaptic, nigral-caudate pathway (Feltz and McKenzie, 1969; Feltz and de Champlain, 1972). The electrophysiological evidence available suggests that caudate neurones can be depressed or facilitated by nigral electrical stimulation (Frigyesi and Purpura, 1967; Connor, 1970) whereas the iontophoretic application of DA predominantly depresses caudate neurone firing rate (Bloom et al., 1965; McLennan and York, 1967). Following large depletions of striatal DA in cats, Feltz and de Champlain reported that nigral stimulation resulted in a long latency excitation of caudate neurones and these workers suggested that the pathway involved was monosynaptic in nature. Therefore there existed a possibility that non-dopaminergic nigral neurones could have been antidromically driven by caudate stimulation thus invalidating this approach in establishing a useful criterion for DA cell recognition.

The selective depression of the zona compacta and VTA neurones by intravenous administration of the DA receptor agonist, apomorphine, was in good agreement with the work of Bunney et al., 1973. All cells having the electrophysiological properties of DA cells were strongly depressed by small doses of apomorphine and in some cases a complete inhibition was observed, albeit a very brief one. In agreement with

Bunney, the recovery period of the DA cell firing rate following apomorphine administration was biphasic in nature. A rapid recovery to about 30% of basal firing rate was followed by a much more gradual recovery phase back to the control level. Control, non-dopaminergic cells located in the substantia nigra, zona reticulata, medial lemniscus and red nucleus were not inhibited by apomorphine and were largely unaffected by this drug. Bunney et al., 1973, included 39 non-dopaminergic cells in their study and found that they either failed to respond or slightly increased their rate response to apomorphine.

The accompanying cardiovascular effects of apomorphine were similar to those found by Finch and Haeusler, 1973, i.e. a short lasting fall in blood pressure and bradycardia. The very rapid inhibition of DA cell firing rate following apomorphine, had a similar time course to the fall in blood pressure. However, the mean blood pressure had almost recovered to its control value within 2-3 minutes while the DA cell firing rate was still strongly depressed. It is unlikely that the effect on DA cell firing rate was secondary to the cardiovascular effects of apomorphine. Additional evidence from administration of haloperidol shows that this dopaminergic receptor antagonist causes a very similar fall in blood pressure but an increase in DA cell firing rate. Intraperitoneal injections of apomorphine are known to completely inhibit DA neurones (Walters, Bunney and Roth, 1975), for an average of 7 minutes and then recover in an identical biphasic manner as that observed with intravenous drugs. Although the blood pressure and heart rate were not monitored in these experiments it seems likely that the doses of apomorphine

used (2 mg./kg.) would have a less pronounced effect on these particular parameters. The hypotensive effect and bradycardia produced by apomorphine are not blocked by haloperidol or spiroperidol (Finch and Haeusler, 1973) suggesting that these effects are not mediated via dopaminergic receptors. In contrast, the results from this study and from Bunney et al., 1973(a), (b), shows that haloperidol can prevent the depressant effect of apomorphine on DA cell firings. This underlines that these effects of dopaminergic drugs on DA cell firing rate are probably mediated via central dopaminergic receptors and are not secondary to cardiovascular effects.

D-amphetamine was found to cause a decrease in DA firing rate but not as marked as apomorphine. Bunney et al., 1973 reported that the mean intravenous dose that inhibited DA cell firing rate by 50% was 1.60 mg./kg. and that there was a considerable variation in the required dosage to cause a given degree of inhibition. The recovery of the DA cell firing rate after d-amphetamine was biphasic; an initial rapid recovery followed by a gradual stage and full recovery, usually within two hours. At the dosage of d-amphetamine used in this study and by Bunney et al., 1973, it is unlikely that the blood pressure of the animal was affected (Foote, Sheard and Aghajanian, 1969). Although the effects of d-amphetamine were not tested on non-dopaminergic cells in this study, Bunney et al., 1973, reported that 29 cells located outside the zona compacta of the substantia nigra and the A10 areas were unaffected by this drug when administered intravenously.

Bunney and Aghajanian, 1973(b) postulated that d-amphetamine

mediated its effect on dopaminergic neurones via a neuronal feedback pathway and supported this by performing brain hemitransections that destroyed the feedback pathway i.e. the striato-nigral projection. Groves and Rebec, 1975, have criticised these lesions on the basis that they would almost certainly damage the dopaminergic nigro-striatal axons. These workers found that intra-nigral injections of d-amphetamine inhibited DA neurones and postulated, in agreement with Aghajanian and Bunney, 1973, that DA receptors were present on the soma of these neurones. Moreover, Groves and Rebec postulated a dendritic release of DA from DA cells and this inhibited other DA neurones or even possibly the same DA neurones. D-amphetamine was suggested to be causing the release of this dendritic store of DA. In a recent paper Aghajanian and Bunney, 1976, have reiterated their neuronal feedback mechanism for the action of d-amphetamine. They claim to have interrupted the striato-nigral pathway by lesioning parts of the internal capsule without damaging the nigro-striatal DA system. However, they do seem to have neglected the findings of Ungerstedt, 1971, who demonstrated that these dopaminergic fibres actually entered the internal capsule before innervating the striatum. Thus, it is likely that these axons were destroyed in these studies. The depressant effect of D-amphetamine on DA neurones was abolished by such lesions while that of apomorphine was not. Aghajanian and Bunney agree with Groves and Rebec that apomorphine acts presynaptically, possibly via DA cell autoreceptors. However, these two groups of workers differ greatly on their explanation as to the mode of action of d-amphetamine on DA neurones and the situation will probably not be resolved until specific lesions of feedback pathways can be made, thus allowing a clear separation of pre- and post-synaptic effects.

The present studies shed no further light on the site of action of apomorphine or amphetamine on the nigral DA system. Presynaptic and/or post-synaptic mechanisms could be involved. In a later section this problem is discussed more fully in the light of biochemical data presented following well-localised striato-nigral pathway lesions. These lesions spared the nigro-striatal DA system. In this section of work it was important to confirm the findings of Bunney et al., and Groves and Rebec and add a pharmacological criterion, in addition to the electrophysiological ones, which would allow DA cell recognition with a greater degree of confidence.

The increase in DA cell spike amplitude following apomorphine administration has not been reported by other workers in the field and it is impossible to check this finding from their published data, since it is presented in the form of reconstructed firing rate histograms with no illustration of oscilloscope traces showing DA cell spikes. Bunney and Aghajanian, 1976, stated that the maximum rate of DA cell firing induced by any drug they have studied was approximately 12 spikes/sec., beyond which the cells appeared to go into "depolarisation block". Earlier these workers also reported that haloperidol caused "DA cell firing characterised by repeated bursts with progressively decreasing spike amplitude". This finding was confirmed in the present investigation. All the cells included in the present study showed an increase in spike amplitude in response to apomorphine and this change was well correlated with the mean interval of the spikes. It is puzzling that other workers have not reported similar observations since the anaesthetic was the only major difference between the present studies and other reported investigations.

Apomorphine, by acting on DA autoreceptors could possibly cause this spike amplitude change simply by slowing the DA cells down. In the gallamine-paralysed, unanaesthetised preparation DA cells fire at a slower rate than in chloral hydrate or halothane anaesthetised animals. Thus, one of the effects of anaesthesia is to speed up DA cells which could possibly cause a slight depolarisation of these cells. DA cells appear to fire in a tightly controlled frequency range and it may be that they are particularly sensitive to firing rate changes. Disinhibition of this system by anaesthesia might possibly cause the cells to become depolarised and this may be reflected in a decreased extracellular spike amplitude. Apomorphine inhibits the DA cells and this in turn might be expected to increase the spike amplitude if one assumes that anaesthesia is causing a slight depolarisation of these cells by speeding them up. This explanation, although parsimonious does appear to fit in with the limited data and further evidence will probably depend on the ability to record intracellularly from identified DA neurones. This approach would be particularly useful in this case because it would allow intracellular potentials to be recorded during the administration of various DA agonists and antagonists.

CHAPTER III

AN INVESTIGATION INTO POSSIBLE AFFERENT PATHWAYS TO THE SUBSTANTIA NIGRA DA-CONTAINING NEURONES USING A COMBINED ELECTROPHYSIOLOGICAL AND NEUROANATOMICAL APPROACH.

INTRODUCTION

Although many studies have centred on the biochemical and pharmacological aspects of the SN DA-containing neurones there is an apparent lack of investigations into the afferent pathways to this system. The existence of a striato-nigral pathway has been known for many years and is the only afferent system to the SN that has been the subject of a number of electrophysiological studies, (Purpura and Frigyesi, 1967; Goswell and Sedgwick, 1971; Yoshida and Precht, 1971; McNair, Sutin and Tsubokawa, 1972; Crossman, Walker and Woodruff, 1973; Frigyesi and Szabo, 1975; Feger and Ohye, 1975; Dray and Gonye, 1975; Dray, Gonye and Oakley, 1976). The consensus of opinion regarding the functional importance of this pathway is that it exerts a predominantly inhibitory influence on SN neurones. Apart from the studies of Dray and his co-workers, little attention has been paid to the accurate localisation of the responsive neurones within the SN. From the reported histological data and electrophysiological characteristics of the recorded neurones it would appear that they were predominantly located in the zona reticulata region. However, Dray et al., 1976, reported that caudate stimulation inhibited a high percentage of neurones in both the zona compacta and zona reticulata areas. A small percentage of kinds of neurones showed excitatory responses to the stimulation. It has also been reported that neurones in all regions of the SN are inhibited by median raphe nucleus stimulation (Dray, Gonye, Oakley and Tanner, 1976) suggesting that there may be a serotonergic input to this area.

In general, however, electrophysiological studies have yielded scant information on other possible inputs to the SN DA system. Part of the problem is the lack of detailed anatomical studies on the afferent inputs to the zona compacta region. However, recent behavioural studies have suggested that nigral DA neurones may be important in sensorimotor function. In order to present a general background in which it was thought to be important to perform the present electrophysiological experiments on the SN DA system, it is necessary to discuss these studies briefly.

The discovery that 6-OHDA had specific neurotoxic effects on central catecholamine neurones (Ungerstedt, 1968, 1971) spurred a great deal of investigations into the behavioural effects of SN DA cell lesions. Unilateral chemonigrectomy produces an initial postural asymmetry in rats, characterised by extension of the limbs contal-lateral to the lesion and turning towards the lesioned side (Ungerstedt, 1971(b)). This asymmetry soon disappears during normal rat activity but it can be elicited when the animals are stressed by tail-pinching or handling. Thus the initial studies indicated that a unilateral lesion had a minimal chronic effect on behaviour, apart from causing a masked motor dysfunction. In contrast, however, bilateral lesions of the nigral dopaminergic system caused a profound behavioural change (Ungerstedt, 1971(d)). The animals became chronically adipsic and aphagic as well as being hypoactive and died unless supported by daily intragastric feeding. This behaviour was closely akin to the "bilateral hypothalamic syndrome" which was observed following bilateral electrocoagulative lesions in the lateral hypothalamus (Anand and Brobeck, 1951; Teitelbaum and Epstein, 1962).

The stereotaxic mapping of the ascending DA axons (Ungerstedt, 1971(a)) revealed that they were present in a dense bundle at the tip of the internal capsule in the far lateral hypothalamic region. This anatomical finding suggested that a lesion of these ascending DA fibres may be involved in producing the "lateral hypothalamic syndrome". In subsequent studies it was shown that 6-OHDA induced lesions in the lateral hypothalamus also produced this severe syndrome, (Ungerstedt, 1971). Damage to the ascending noradrenergic system caudal to the substantia nigra failed to produce aphagic or adipsic symptoms. In addition, lesions of the limbic DA fibres only produced a short period of hypodipsia and hypophagia. Thus, Ungerstedt 1971, postulated that the complete destruction of the nigral DA system, in particular, the nigro-striatal system, was responsible for the "lateral hypothalamic syndrome". This suggestion has been confirmed by other workers, who have achieved nigral DA depletion by intra-ventricular as well as intracerebral administration of 6-OHDA (Zigmond and Stricker, 1972; Fibiger, Zis and McGeer, 1973; Stricker and Zigmond, 1974; Stricker, Frieman and Zigmond, 1975). In addition, all these workers have reported that the DA lesioned animals gradually recover and are able to restart feeding and drinking but that there are persistent deficits in response to various regulatory challenges which normally increase food intake e.g. cold ambient temperature. One important feature of the reported DA lesion studies is that the severity of the syndrome seems to be well correlated with the extent of the DA depletion (Ungerstedt, 1973) and that compensatory mechanisms occur in the remaining DA neurones and receptors so that almost normal DA function is possible (Stricker and Zigmond, 1976).

Although the "lateral hypothalamic syndrome" has been known for over 20 years it is only recently that reports have appeared which may explain the functional deficits present in this syndrome. Behavioural studies in rats with unilateral hypothalamic lesions showed that these animals failed to orientate to contralateral, visual, olfactory, whisker touch or somatosensory stimulation (Marshall, Turner and Teitelbaum, 1971). This finding was confirmed by Turner, 1973, who suggested that the unilateral sensory neglect was neither a primary sensory nor motor deficit but rather a disruption of the linkage between contralateral sensory and motor systems. Bilateral 6-OHDA-induced lesions in the nigro-striatal bundle also produce similar sensorimotor impairments but bilaterally (Marshall, Teitelbaum and Richardson, 1974). These initial severe impairments do gradually recover but at different post-lesion times (Marshall and Teitelbaum, 1974). A nigral lesioned animal may respond to olfactory and visual stimulation by orientating towards it but be totally unresponsive to tactile stimulation. This underlines the fact that the primary deficit is not in the animals motor capability. Although these behavioural tests are relatively crude they do serve to demonstrate that nigral DA lesions do seem to initially impair sensorimotor function. The importance of these sensorimotor deficits in the aphagia, adipsia and inability to regulate food and water intake is not clear but it does seem likely that they at least are responsible for the initial stages of the LH syndrome.

These chemical lesion studies have provided the most convincing evidence so far that the DA system may be important in sensorimotor function. However, other investigations, especially in the field of

intracranial self-stimulation have reached similar conclusions (Crow, 1972; Crow and Arbuthnott, 1972; Routtenberg 1974). It is known that many sites in the ventral mesencephalon and brainstem support self-stimulation (Routtenberg and Malsbury, 1969; Huang and Routtenberg, 1971; Crow 1971; Crow 1972) and on the basis of these positive sites Crow 1972 postulated that brain catecholamine neurones were important for the mediation of this behaviour. This hypothesis was strongly supported by pharmacological data which showed that self-stimulation behaviour could be enhanced by amphetamine (Crow 1969; Wise and Stein 1970) or decreased by catecholamine depleting agents such as alpha-methyl- para tyrosine (Poschel and Nintemann 1966; Gibson and McGeer and McGeer, 1970; Black and Cooper, 1970). Crow suggested that both noradrenaline and dopamine were important in self-stimulation behaviour and that either catecholamine system could support it independently of the other. The NA system arising in the locus coeruleus, was suggested to be a "reinforcement centre", whereas the DA system in the SN was suggested to be an "incentive motivational centre". The essence of Crow's hypothesis was that these two catecholamine systems were intimately related to the central areas of gustation (NA) and olfaction (DA). For the DA system Crow argued "that there exists an association between the DA-containing neurones and the central connections of olfaction, such that the DA neurones constitute a final link in the pathway which mediates the general effects of positively rewarding olfactory stimuli. In the course of evolution other inputs have come to dominate this system so that it commonly is activated by stimuli (in modalities other than olfaction) which by

previous learning have acquired significance for the organism. This mechanism is presumed to be at the basis of "incentive motivational effects". Thus, Crow visualised the DA system as having primarily a motor function and he emphasised the importance of the "reward" nature of sensory information.

The anatomical evidence in support of Crow's hypothesis is very tenuous and relies more on the anatomical juxtaposition of the DA system in the SN and the olfactory system than on possible functional connections between the two. Crow thought it possible that olfactory information could reach the DA system by two possible routes; either via the habenular-interpeduncular pathway or by descending fibres of the medial forebrain bundle. With regard to the first possibility, it is known that olfactory information reaches the habenular nucleus (Herrick, 1948; Cajal, 1966; Rausch and Long, 1971; Wedgewood, 1974; Mok and Mogenson, 1974) and that the major efferent pathway from this nucleus projects to the interpeduncular nucleus (Cragg, 1961; Smaha and Kaelber, 1967; Cajal, 1966; Akagi and Powell 1968; Lake, 1973). The DA neurones in the A10 and A9 groups surround this nucleus and Crow thought it possible that these neurones were likely to be dominated primarily by olfactory input. The descending fibres of the medial forebrain bundle, terminating perhaps in the ventral tegmental area surrounding the interpeduncular nucleus, was the second possible route by which olfactory information could reach the DA system. Evidence for direct olfactory projections to the SN does not exist in anatomical terms although it is possible that functional, polysynaptic pathways do exist. The attraction of this hypothesis was that it was perhaps testable electrophysiologically.

Routtenberg, 1974, also tried to define neuronal pathways that could possibly act as substrates for self-stimulation behaviour. His approach, as outlined by Huang and Routtenberg, 1971, was to place discrete lesions through implanted electrodes that had been previously shown to support self-stimulation behaviour and trace the resulting degeneration pattern using the Fink-Heimer technique. On the basis of these results microelectrode studies were performed to investigate the responsiveness of neurones in different brain areas to electrical stimulation applied through the implanted electrodes. Routtenberg did not suggest that catecholamines were important in self-stimulation behaviour but rather that anatomical connections did exist between distant positive sites and electrical stimulation at various points along these pathways could support this behaviour. He therefore proposed a stimulus- response organisation of self-stimulation and tentatively suggested a four- stage model to account for his experimental findings. The model is complex and includes many pathways based on questionable anatomical findings. However, he does report in his electrophysiological data that cells in the lateral hypothalamic area project to cells in the zona compacta region of the SN and suppress their firing rate. In general terms Routtenberg proposed that the extra pyramidal system, including the zona compacta region could be influenced by all kinds of sensory information, rewarding or otherwise.

In support of both the Crow and Routtenberg hypotheses, earlier self-stimulation studies had suggested that this behaviour was due to activation of afferent pathways (Campbell, 1968; Phillips and Mogenson, 1969). In view of the lack of anatomical and electrophysiological

studies on the afferent connections to the SN DA system, the hypothesis forwarded by Crow has had a considerable influence on the design of the present experiments. Since no studies of any kind have either confirmed or disproved this hypothesis, the present experiments were undertaken to examine the effects of electrical stimulation of various brain areas associated with olfaction upon the activity of single substantia nigra neurones. In addition, the possibility of a lateral hypothalamus- zona compacta pathway was investigated. Anatomical pathway tracing techniques have been used in an attempt to try and establish possible neural connections projecting from olfactory areas and the lateral hypothalamus to the DA system in the SN.

METHODS

1. Electrophysiological recording of SN neurones and Electrical Stimulation of the Brain.

Male albino male rats (n=50) in the weight range of 190-210 grams were anaesthetised with 0.7% halothane/air mixture and single units in the substantia nigra were recorded in an identical manner to that described in detail in the previous chapter. For electrical stimulation bipolar concentric stainless steel electrodes (outer diameter 0.5mm. : tip separation 0.3-0.5mm.) were placed stereotaxically in one of the following brain areas; ipsilateral olfactory bulb, anterior olfactory nucleus (ipsilateral), medial and lateral habenular nucleus (ipsilateral). The coordinates for electrode placements in each of the brain areas were as follows:

	Olfactory bulb	Habenular nucleus	Anterior Olfactory nucleus
A- P	5.5	4.3	3
L	2.2	1.4	4
V	3.5	5.2	0.6 - 0.8

All coordinates are in millimetres. The A-P and L coordinates were with reference to the bregma suture and the vertical reading was taken from the overlying cortical surface in each case.

Single pulse stimulation was used most frequently, 0.2 - 1 Hz. being the most common frequency of stimulation. In some instances repetetive train stimulation was used. The stimulation train lasted between 7-10 msecs and consisted of 3-10 pulses. All pulses of stimulation had a duration of 0.1 msec. A Digitimer (Devices

Instruments Ltd.) was used to initiate periods of stimulation generated by an isolated stimulator (Devices). The stimulation voltages used in the following experiments were in the range 5-60 V which corresponded to stimulation currents of 0.05 - 0.7 mA as measured by the potential drop across a 100 Ohm resistor placed in series in the stimulation circuit. Poststimulus responses of neurones were displayed on a storage oscilloscope (Tektronix D-13 Dual Beam Storage) and poststimulus histograms were obtained and analysed using a Biomac 1000 computer.

Stimulation of the anterior olfactory nucleus was found to evoke a potential in the contralateral olfactory bulb. This slow potential was recorded from the exposed olfactory bulb surface by means of a fine silver wire and amplified by a Tektronix 5A22N amplifier and displayed on a Tektronix D13 oscilloscope. This potential was used to give an indication of the correct stimulating electrode placement in the anterior olfactory nucleus.

2. Lesioning and Histological Procedures

(a) Electrolytic lesioning of the habenular nucleus

12 adult, male Wistar rats, weighing 190-210 grams were anaesthetised with 3% halothane/air mixture and their heads were fixed in a stereotaxic instrument (David Kopf Instrument) and anaesthesia maintained by a 1% Halothane/air mixture. A 001 insect pin, 0.2mm. in tip diameter and insulated to within 0.5mm. of the tip, was stereotaxically positioned in the habenular nucleus according to the following coordinates:

Posterior	3.1mm.
Lateral	0.7mm.
Vertical	4.0mm.

The bregma suture was used as the stereotaxic reference point for the anterior-posterior and lateral coordinates and the overlying cortical surface was the zero reading for the vertical coordinate. Electrolytic lesioning of this nucleus was performed with a 12V power supply by passing a current of 2mA for 6 secs. Following the lesioning procedure antibacterial agent (Polybactrin) was applied to the operated area to prevent subsequent infection. The incision in the scalp was carefully sutured and the animal was allowed to recover.

(b) Modification of the Fink-Heimer Technique for Silver Impregnation of Degenerating Axons and Terminals in Fixed Brains.

The method used was that described by Hjorth-Simonsen, 1970. This technique was essentially the silver impregnation method of Fink and Heimer, 1967, applied to cryostat sections of formalin-fixed brain tissue, mounted on slides, instead of on free-floating frozen sections processed individually.

The optimum survival time, following habenular nucleus lesions was found to be 4 days. Lesioned animals were deeply anaesthetised with chloral hydrate 400 mg./kg. i.p. and transcardially perfused with 80ml. of physiological saline followed by 250ml. of 10% formalin made by a 9:1 dilution of a stock solution (38% HCHO) which had been previously neutralised by saturation with $CaCO_3$ (marble chips). Following perfusion the brains were quickly removed from the skull and placed in 10% formalin solution for a further 1-2 weeks.

Thereafter, the brains were soaked in a 25% weight/volume (w/v) sucrose solution for 2-3 days and then different brain areas, sectioned coronally and 4-5mm. in thickness, were frozen by CO_2 expansion and sectioned at 20 microns at a temperature of -25° C.

It was found that fixed sections do not readily adhere to unsubbed slides so it was necessary to coat glass slides with a chrome alum- gelatin solution prepared as follows: 0.3 grams of chrome alum was dissolved in 600 ml. of 0.5% aqueous gelatin solution and clean glass slides were dipped into this solution and allowed to drain and dry in an oven maintained at 40° C.

After drying, the coated slides were cooled in a cryostat and selected brain sections, taken at 100 microns through the mid brain, were mounted on to them. The sections were dried at room temperature for at least two hours. It was important to use slides within 24 hours of the coating procedure to avoid subsequent non- specific silver deposits.

The silver impregnation stage was carried out in glass staining dishes. All sections were treated according to the steps outlined by Fink and Heimer, 1967.

a. Immersion in 0.025% potassium permanganate solution for 5 minutes
b. Rinsed with distilled water and bleached for 60 seconds in a freshly prepared 1:1 mixture of 1% oxalic acid and 1% hydroquinone.
c. Thorough rinsing in distilled water (5 minutes) and immersed in a fresh uranyl nitrate solution; 2.5% aqueous, for 5 minutes.

d. Washed for 5 minutes in 2 changes of distilled water and impregnated for 2-6 hours at 37°C. in 0.2% silver nitrate solution, containing 2% pyridine.

e. The sections were then impregnated 5 minutes in a solution containing 1.5% silver nitrate, 100 ml.; 96% ethanol, 60 ml.; concentrated NH_4OH, 10 ml.; and 2.5% NaOH, 8.5 ml., mixed in the order given.

f. The sections were then transferred, without rinsing or blotting to a Nauta reducing solution consisting of: water, 900 ml.; absolute ethanol, 100 ml.; 1% citric acid, 30 ml.; and 10% formalin, 30 ml.. Two changes were used, 5 seconds in the first and 2 minutes in the second.

The published method stressed that proper timing at this stage was critical to avoid non-specific silver deposits.

Finally, the sections were rinsed with distilled water, dehydrated in increasing concentrations of ethanol, 50-100% and cleared in a mixture of: xylene, 80ml.; creosote, 10ml.; and phenol, 10 grams. All sections were mounted in Canada balsam.

Sections were examined under a light microscope, using a x40 objective. The distribution of deposited silver grains was carefully plotted and superimposed on different planes of section taken from the atlas of König and Klippel, 1963.

In all cases adjacent sections were collected and stained according to the method of Klüver and Barrera (1953).

3. Autoradiographic Tracing of the Efferent Connections of the Anterior Olfactory Nucleus and the Lateral Hypothalamus

The technique used throughout this study was based on the general approach outlined by Cowan, Gottlieb, Hendrickson, Price and Woolsey, 1972, for the use of certain radioactively labelled amino acids in establishing neural connectivity in the central nervous system. In the present study the technique used was similar in detail to that reported by Pickel, Segal and Bloom, 1974.

a. Preparation and Intra-cerebral Injection of L- 4,5-^{3}H leucine

1 mCurie of L-4,5-^{3}H leucine (Radiochemicals, Amersham) in a 1ml. aqueous solution containing 2% ethanol was dried in a stream of nitrogen until all the liquid had evaporated. The dry residue was redissolved in sterile physiological saline to a final dilution of 20-25 microCuries/microlitre.

In the experiments to be described radioactive leucine was injected in the region of the anterior olfactory nucleus in 6 male Wistar rats 190-210 grams and in the lateral hypothalamic area in a further 12 animals. Each animal was anaesthetised with 1% halothane/ air mixture and placed in a David Kopf stereotaxic instrument. The labelled solution was delivered through a glass micropipette with a tip diameter of approximately 50 μm. The micropipette was attached to a 1ul. Hamilton syringe and stereotaxically placed in the appropriate brain area. In the case of the anterior olfactory nucleus (AON) 0.5 μl. of the labelled solution was injected manually over a period of 30 minutes and the micropipette was held in position for a further

30 minutes. The bregma suture was used throughout as the stereotaxic reference point. The coordinates used for the AON were as follows:

Anterior	4.1 mm.
Lateral	1.6 mm.
Vertical	5.0 mm. (cortical surface was the reference point)

More discrete injections were required in the lateral hypothalamic area studies, therefore, a smaller volume of labelled solution, 0.2 - 0.3 µl., was delivered in an identical manner to that outlined for the AON. The coordinates used in this instance were as follows:

Posterior	2-2.5 mm.
Lateral	1.5 mm.
Vertical	7.8 mm. (cortical surface was the reference point).

b. Post-injection, fixation and histological processing

Following post-injection survival times of 6 hours to two days the animals were deeply anaesthetised with chloral hydrate 400 mg./kg. and perfused through the ascending aorta with 80 ml. of 0.1 M phosphate buffer, followed by 250 ml. of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2). Throughout the perfusion the animals were kept in an ice bath. The brains were then carefully removed from the skull and placed in fresh fixative for an additional hour. Thereafter, the tissue was post-fixed in Bouin's fixative, dehydrated through an increasing series of ethanol solutions 50-100%, cleared in xylene and vacuum embedded in paraffin wax (M.P. 54°C.). The brains were subsequently sectioned at 6 microns and the sections were then floated

on to clean, chrome alum- gelatin coated microscope slides. Before autoradiographic procedures, the sections were deparaffinised in xylene and rehydrated through a series of graded ethanols and finally washed for several hours in running water.

c. Autoradiographic procedures

All procedures were carried out in a dark-room maintained at a relative humidity of 65% and illuminated by a 25W lamp with a Kodak Wratten filter No.1. For light microscope autoradiography Kodak AR10 stripping film was used to coat all sections.

Small pieces of AR10 film were carefully floated on the surface of a particle-free solution of potassium bromide (1%) and sucrose (2%) maintained at a temperature of 22-24°C. After a floating time of 2 minutes, the expanded and crinkle-free film was draped around the glass slides and the coated slides were then hung up to dry for 3 hours. After drying the slides were packed in light-tight boxes and stored in a freezer held at -20°C and exposed for 30 days.

The exposed slides were subsequently developed for 5 minutes in a freshly prepared, undiluted Kodak D-19 developer. The temperature of the developer was maintained between 14-18°C. After rinsing in running water, the photographic emulsion was fixed in Kodak "Metafix" and then washed in gently running tap water for at least 30 minutes. It was found, using this procedure, that the stripping film adhered well to the glass slides and did not crinkle.

All developed slides were subsequently stained through the photographic emulsion with haemotoxylin and eosin. The sections were examined under both light and dark- field illumination and the distribution of silver grains was plotted on appropriate planes of brain section. In all cases a second independent observer verified the grain distribution. Control sections were obtained from non-injected brains which had been processed in an identical manner. These sections served as an indication of whether a given batch of slides had a sufficiently low background level to be of use in pathway mapping.

RESULTS

Projection of the Habenulo- interpeduncular pathway

The purpose of this anatomical tracing experiment was to investigate the detailed projections of the habenular nucleus to midbrain areas. The modified Fink-Heimer method was found to give very clear results since non-specific background staining was very well suppressed. However, it was important that the sections were relatively free from cryostat knife marks since these tended to stain with a dark granular appearance which could possibly lead to confusing results. Stained sections had a yellow to golden-yellow background and terminal degeneration was indicated by the presence of characteristic "dust-like" black grains. Axonal degeneration was, on the whole much rougher in appearance and consisted of larger grains. Nevertheless, it was difficult to distinguish between the two types of degeneration, especially when both were present in the same area.

Lesions were successfully placed in the habenular nucleus of 4 animals. All the lesions were found to be well localised to this nucleus and only minimal damage occurred in the surrounding brain areas. The neuronal degeneration pattern was similar in all four animals studied and the results from one animal are shown in Fig.18. In this particular case the electrolytic lesion ablated the entire lateral habenular nucleus and a substantial part of the medial habenular nucleus (Fig.19).

Ventrally, dense axonal degeneration was observed throughout the entire extent of the ipsilateral habenulo-interpeduncular tract,

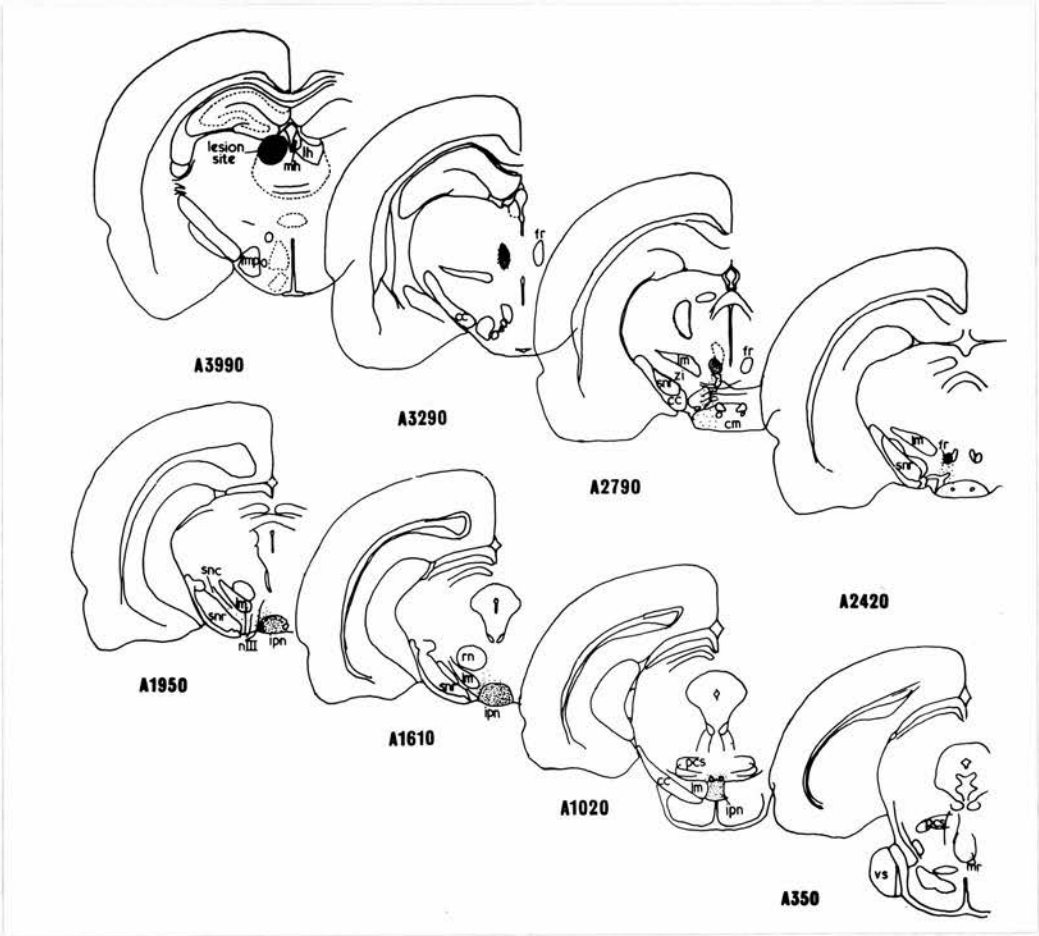

Figure 18

Schematic diagram showing habenular nucleus lesion site and the distribution of degenerating fibres and terminals as demonstrated by a modified Fink-Heimer technique. The post-lesion survival time was 4 days. The numbers below the planes of sections refer to the anterior-posterior planes of section taken from the atlas of König and Klippel. Note that the fasciculus retroflexus, ipsilateral to the lesion, is completely shaded in because of the intensity of degeneration observed in this tract. Heavy terminal degeneration was found throughout the interpeduncular nucleus (denoted by intensity of black dots).

Abbreviations: cc, crus cerebri; cm, corpus mammillare; fmp, medial forebrain bundle; fr, fasciculus retroflexus; ipn, interpeduncular nucleus; lh, lateral habenular nucleus; lm, lemniscus medialis; mh, medial habenular nucleus; nIII, oculomotor nerve; pcs, pedunculus cerebellaris superior; rn, red nucleus; snc, substantia nigra, zona compacta region; snr, substantia nigra, zona reticulata region, vs, fifth cranial nerve.

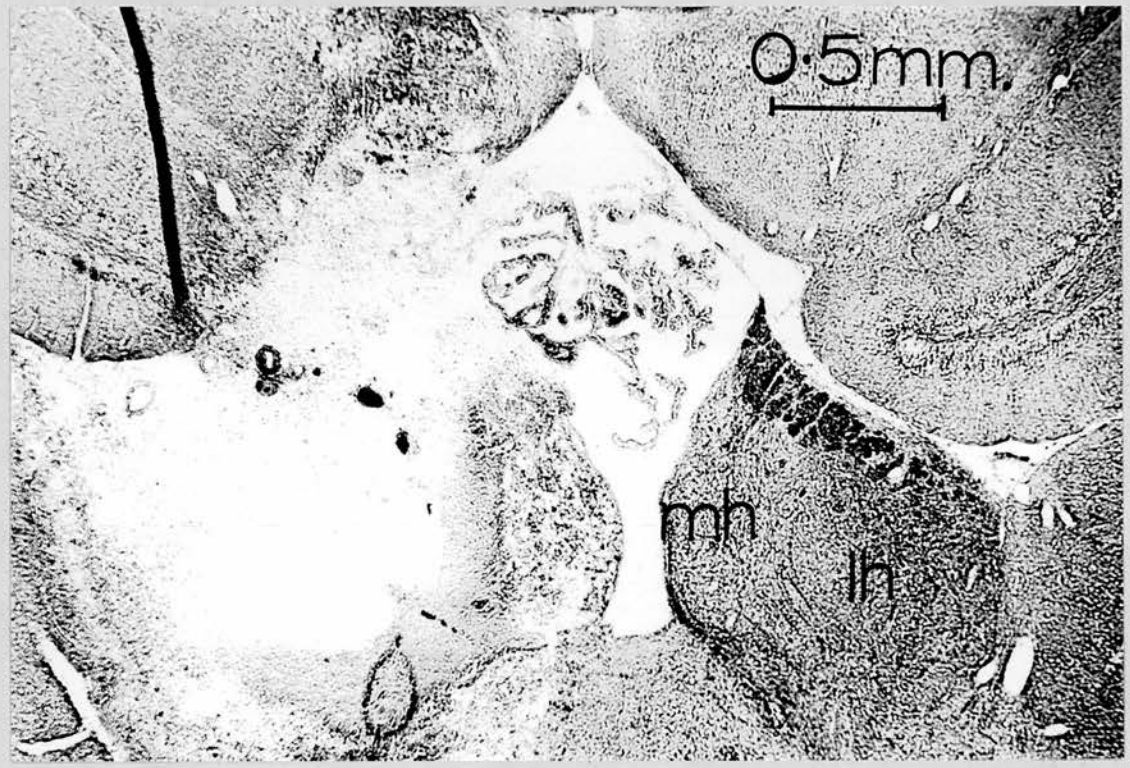

Figure 19

A photomicrograph showing the complete, unilateral ablation of the lateral habenular nucleus and partial ablation of the medial habenular nucleus, following an electrolytic lesion produced by the passage of 2mA current for 6 secs. The section was stained with luxol fast blue and cresyl violet.

Abbreviations: mh, medial habenular nucleus; lh, lateral habenular nucleus.

termed the fasciculus retroflexus in Fig.18. A few degenerating terminals were observed in the ipsilateral region of the mammillary bodies. The heavily stained habenulo-interpeduncular tract coursed ventrally to the lateral margin of the interpeduncular nucleus and degenerating fibres could be seen entering this nucleus. Heavy terminal degeneration was found throughout this nucleus (Fig.20). In addition a few grains were observed in the ventral tegmental area and medial zona compacta. However, this degeneration was very sparse and it is uncertain whether it was terminal or axonal in origin. In the most caudal plane of section studied, light degeneration was well localised in the region of the medial raphe nucleus.

It should be noted that a few myelinated nerves appeared to be darkly stained. This staining was quite distinct from terminal and axonal degeneration and consisted of strands of dark brown material. It was also much less intense than the specific staining found in degenerating nerves. Fig.21 shows a few fibre bundles of the third cranial nerve and ventral tegmental decussation coursing through the medial-posterior region of the substantia nigra.

Brain areas outwith the planes of section shown in Fig.18 were not studied in this investigation but it is known, from studies on the cat, that efferent pathways do project to rostral areas not included here (Akagi and Powell, 1968).

Electrical Stimulation of the Habenular Nucleus: Effect on Neurones Located in the Substantia Nigra

The major difficulty encountered in this investigation was the

Figure 20

Photomicrograph from the region of the caudal interpeduncular nucleus taken 4 days after an electrolytic lesion which completely ablated the lateral habenular nucleus and largely ablated the medial habenular nucleus (shown in Fig.19). The section was stained according to a modified Fink-Heimer technique (Hjorth-Simonsen A. 1970). Note the dense concentration of fine, black grains, characteristic of terminal degeneration, which was found in this region. Scale bar; 20µm.

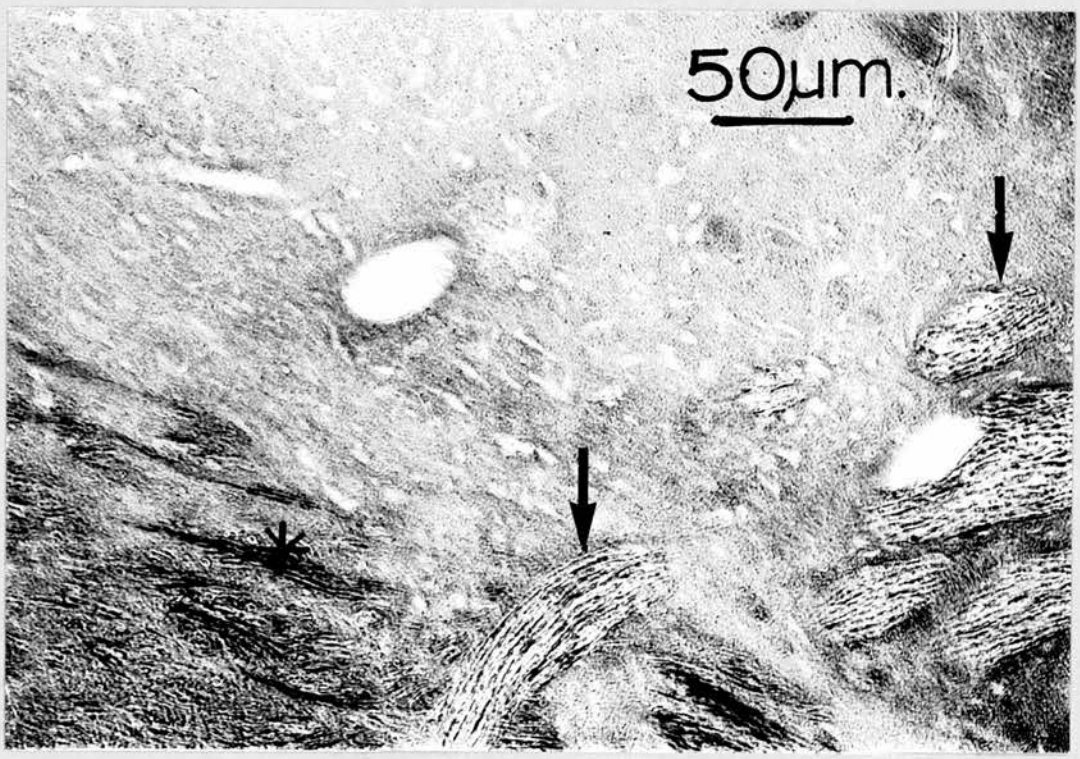

Figure 21

Photomicrograph showing fibre bundles of the third cranial nerve coursing through the medio-posterior region of the SN. The fibre bundles (shown by arrows) appeared to be stained dark brown in the histological material. Fibres of the ventral tegmental decussation (*) also appear stained. This section was stained according to a modified Fink-Heimer technique. (Hjorth-Simonsen, 1970)

correct positioning of the stimulating electrode. No convenient method existed whereby correct placement could be verified prior to recording single cells in the SN. This resulted in 35% of the experiments being discarded. In the remainder the responsiveness of 25 substantia nigra neurones was tested during single pulse and train stimulation of the ipsilateral habenular nucleus. A typical lateral habenular nucleus stimulating site is shown in Fig.22. The recording sites within the SN; 19 in the zona compacta and 6 in the zona reticulata, were plotted onto the appropriate brain section and the summary diagram is shown in Fig.23. All the cells located in the zona compacta area had electrical characteristics that were identical to those outlined in detail in the previous chapter. None of the SN neurones were found to be responsive to habenular nucleus stimulation, either in the medial or lateral aspects. For each cell studied the stimulation current used ranged from 50μA to 0.7mA. In only one instance, during recording a cell in close proximity to the interpeduncular nucleus, was a slow potential recorded. This potential and the single cell record are shown in Fig.24. The stimulation threshold for this slow potential was 150 μA.

Substantia Nigra Neurones and Olfactory Bulb Stimulation

Olfactory bulb electrical stimulation has been used in many studies on the olfactory system. This study, in common with other olfactory studies on the higher centres of olfaction (Scott and Pfaffmann, 1967; Pfaff and Pfaffmann, 1969; Komisaruk and Beyer, 1972; Motokizawa, 1974) made use of gross electrical stimulation of the olfactory bulb with the aim of stimulating the olfactory bulb efferent pathway, the lateral olfactory tract.

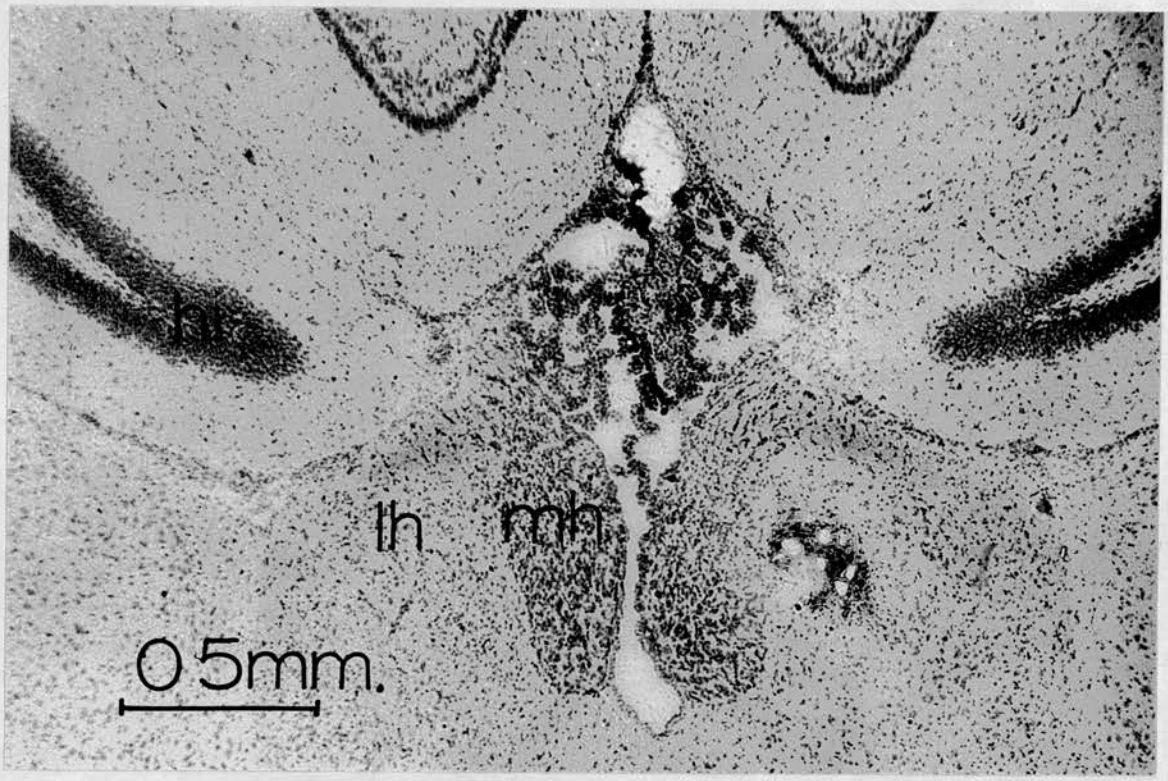

Figure 22

Photomicrograph showing lesion produced at the tip of a bipolar stimulating electrode, following electrical stimulation in the lateral habenular nucleus. The lesion site is indicated by the arrow. Staining is luxol fast blue and cresyl violet.

Abbreviations: hi, hippocampus; lh, lateral habenular nucleus; mh, medial habenular nucleus.

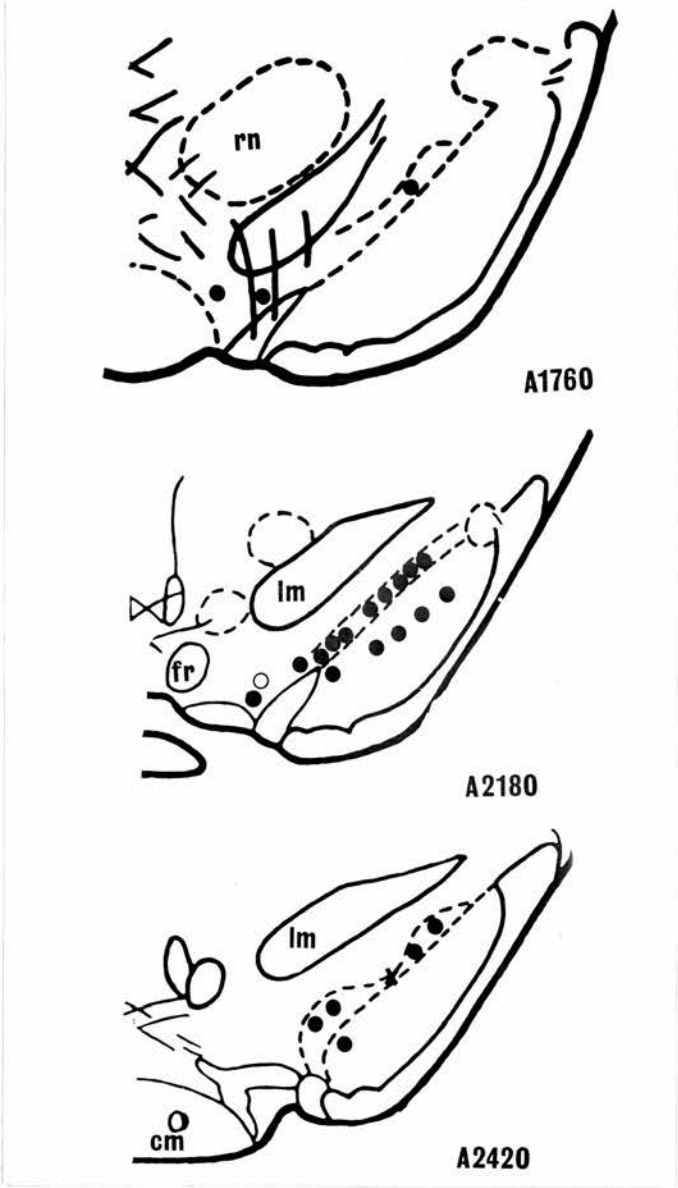

Figure 23

Summary diagram of recording sites in the SN from which neurones were tested for their responsiveness to electrical stimulation of the habenular nucleus. The firing rates of all the neurones studied were unaffected by both single pulse and train stimulation (50-700μA). The recording sites were projected onto the appropriate plane of section taken from the atlas of König and Klippel, 1963. Abbreviations: cm, corpus mammillare; lm, lemniscus medialis; rn, red nucleus.

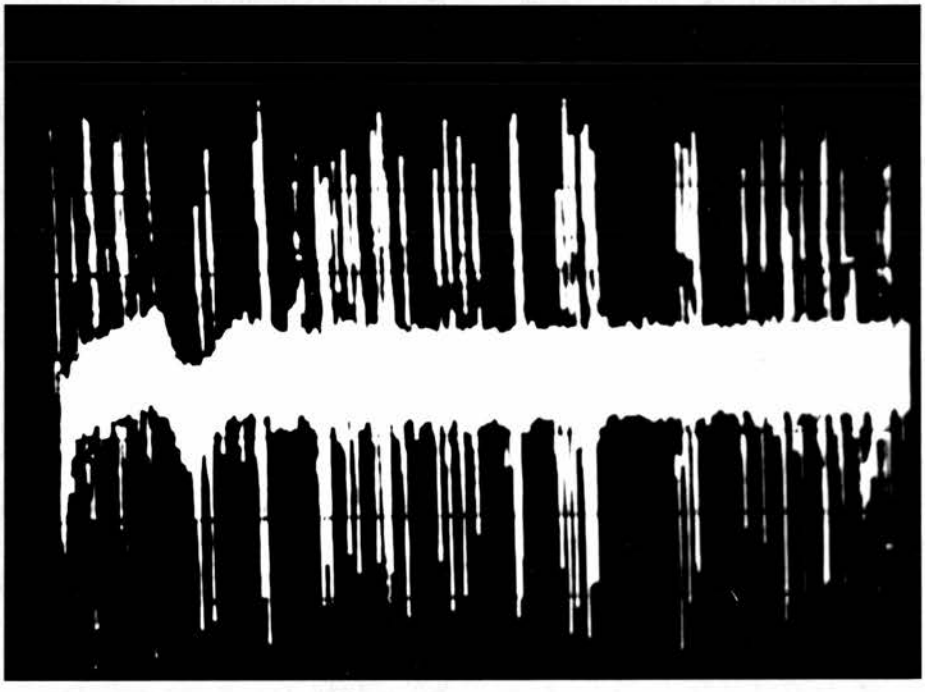

Figure 24

Failure of lateral habenular nucleus stimulation to influence the firing rate of a characteristic DA-containing neurone in the A10 region. The figure is an a.c record of the neurone which was photographed from a stored oscilloscope record of 10 consecutive sweeps. Stimulation was single pulse (0.5Hz) and the stimulating current was 500μA. A slow potential was present at the beginning of the record. The stimulus initiated each sweep and the calibration is given in the figure.

17 neurones, located in the SN, were tested for their responsiveness to olfactory bulb stimulation. 7 of these neurones were located in the zona compacta region and all had the electrical characteristics of presumed DA cells. A summary diagram of all the SN recording sites is shown in Fig.25. None of these cells were found to be responsive to olfactory bulb stimulation or to light foot shock, applied to the ipsilateral or contralateral hind limb. Similarly, all the cells in the zona reticulata were also unresponsive to olfactory bulb stimulation. Stimulating currents in the range 50μA to 200μA were used throughout this study and in no instance was the preparation found to convulse.

Effect of electrical stimulation of the anterior olfactory nucleus On the firing rate of SN neurones

The cells of the main olfactory bulb project partly to the ipsilateral anterior olfactory nucleus. As is shown in the following study on the efferent projections of the anterior olfactory nucleus, the cells in this region give rise to the fibres of the anterior limb of the anterior commissure, the second efferent olfactory pathway relaying sensory information to higher olfactory centres. Thus, it was thought worthwhile to stimulate this pathway in the present experiments.

Stimulating electrodes were placed in the anterior olfactory nucleus within the region shown in Fig.26. Following stimulation of the AON, it was possible to record an evoked potential from the surface of the contralateral olfactory bulb. This potential was initially positive in polarity, followed by a negative phase.

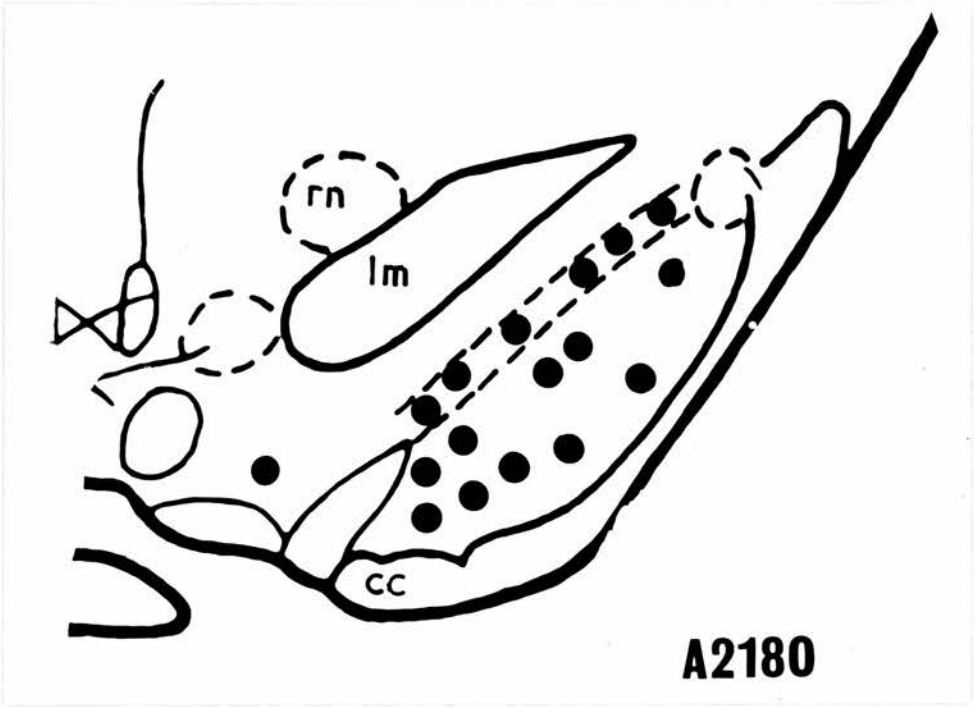

Figure 25

Schematic diagram of electrode recording sites at which neurones were tested for their responsiveness to electrical stimulation of the ipsilateral olfactory bulb. The firing rates of all the neurones studied were unaffected by both single pulse and train stimulation (50-300 μA). The section was taken from the atlas of König and Klippel, 1963.

Abbreviations: cc, crus cerebri; lm, lemniscus medialis; rn, red nucleus.

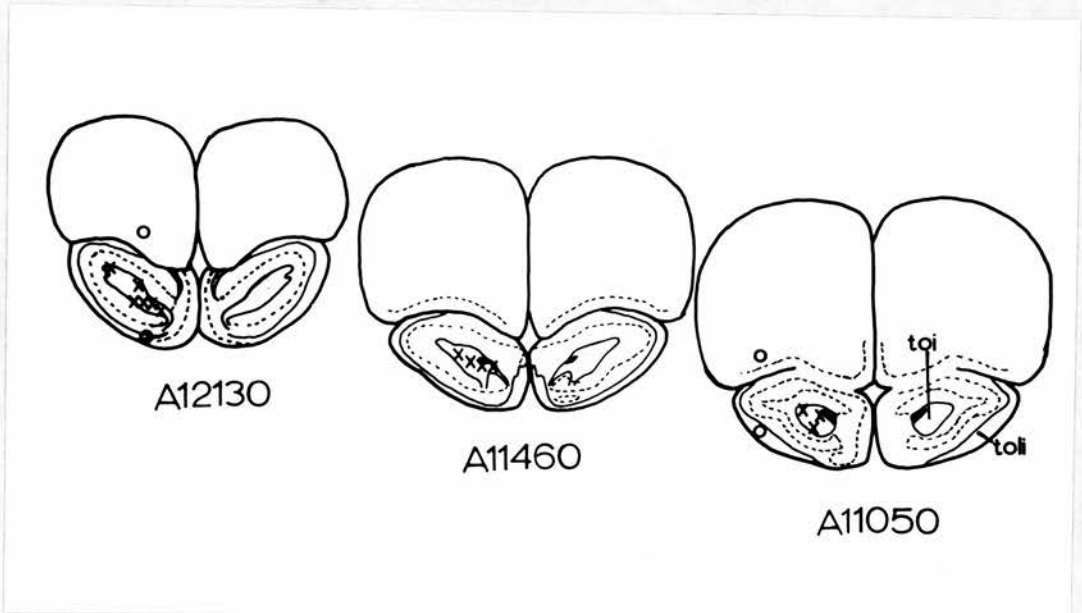

Figure 26

Summary diagram of electrode stimulating sites in the anterior olfactory nucleus. (X) denotes sites at which electrical stimulation evoked a slow potential in the contralateral olfactory bulb and also influenced the firing rate of neurones in the SN. (0) denotes sites at which electrical stimulation failed to evoke a slow potential in the olfactory bulb and also did not influence SN neurone firing rate.

Typical evoked potentials are illustrated in the oscilloscope traces shown in Figs.29 and 30. No detailed study of this evoked potential was made. However, it was observed that when the stimulating electrode was placed centrally in the AON, the threshold stimulation current required to evoke this potential was in the range 150 to 250μA. The effect of electrical stimulation of the AON on 29 histologically and electrophysiologically identified zona compacta and zona reticulata neurones was analysed. A summary diagram of these recording sites is shown in Fig.27. A single lesion site, situated in the zona compacta region is shown in Fig.28. Following single pulse stimulation, the activity of 20 (70%) cells was changed. Of these 20 units, 4 showed an increase in firing rate, 2 showed a decrease followed by an increase, 11 showed a decrease, 1 showed an increase followed by an increase and 2 showed a very complex response involving an increase-decrease-increase sequence. Examples of the facilitatory and inhibitory effects of AON stimulation on zona compacta neurones are shown in Figs.29 and 30. A summary diagram, showing the latency and duration of the responses was constructed from the data available from the post-stimulus histogram traces of individual responsive cells and is shown in Fig.31. The latencies to activation of the neurones was variable, ranging from 8.5msecs. to 32msecs. The latencies of inhibition ranged from 20msecs-120msecs. All these effects, both facilitatory and inhibitory, showed considerable variation of duration of effect, ranging from 15msecs-200msecs. There was also a wide range of stimulation current intensities required to elicit these responses; from 150μA to 0.7mA.

Recordings were also made from 30 zona reticulata cells, 9 of which were marked by discrete lesions following recording. The remainder were isolated during identified electrode penetrations through the zona reticulata area. Only 2 cells in this neurone population responded to AON stimulation. Both showed a decrease in cell firing rate followed by an increase. These cell responses are also included in Fig.31.

All the cells studied in the substantia nigra were tested for their responsiveness to non-olfactory stimuli. Foot shock was found to elicit responses in 4 neurones, located in the zona compacta region. Three of these cells responded with a decrease in firing rate with latencies to onset of 29-34msecs. 1 cell responded with an increase in firing rate with a latency of 20msecs. These cell responses are included in Fig.31.

In a few experiments attempts were made to test the odour-responsiveness of substantia nigra neurones. However, in no instances were evoked potentials recorded from the olfactory bulb following the application of xylene and amyl acetate vapours to the nares. This suggested that the olfactory receptors on the sensory epithelium were not being activated by these odours. Therefore, these experiments were discarded due to doubts about the olfactory stimulation.

Autoradiographic Tracing of the Efferent Pathways of the Anterior Olfactory Nucleus

In the rat the olfactory peduncle extends from the caudal region of the main olfactory bulb to the prepyriform cortex posterio-laterally.

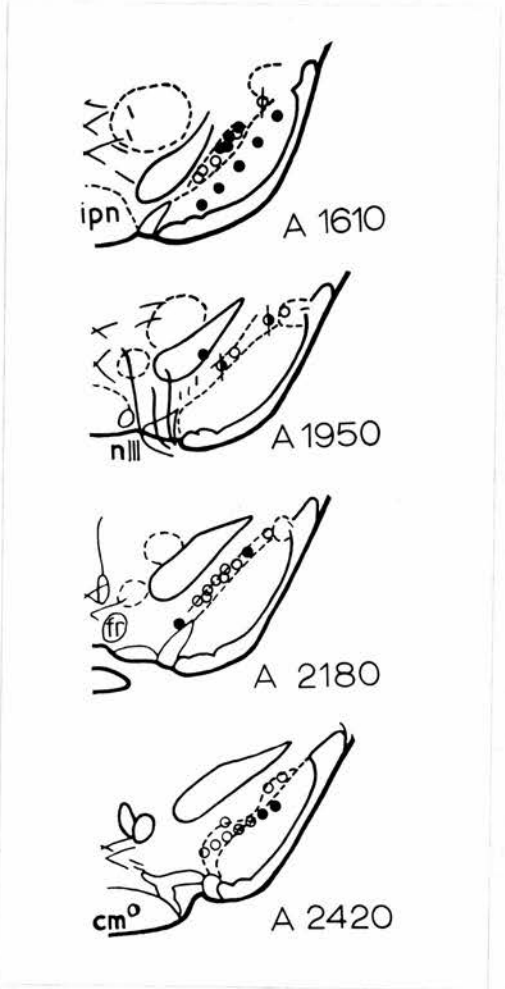

Figure 27

Summary diagram of recording sites in the SN from which neurones were tested for their reponsiveness to electrical stimulation of the anterior olfactory nucleus. The filled circles (●) denote the position of unresponsive neurones; the empty circles (○) denote responsive cells and the half-filled circles (⌀) denote cells responsive to foot shock stimulation. All the recording sites were projected onto the appropriate planes of section taken from the atlas of König and Klippel (indicated by the number under each section) Abbreviations: cm, corpus mammillare; fr, fasciculus retroflexus; ipn, interpeduncular nucleus; nIII, oculomotor nerve.

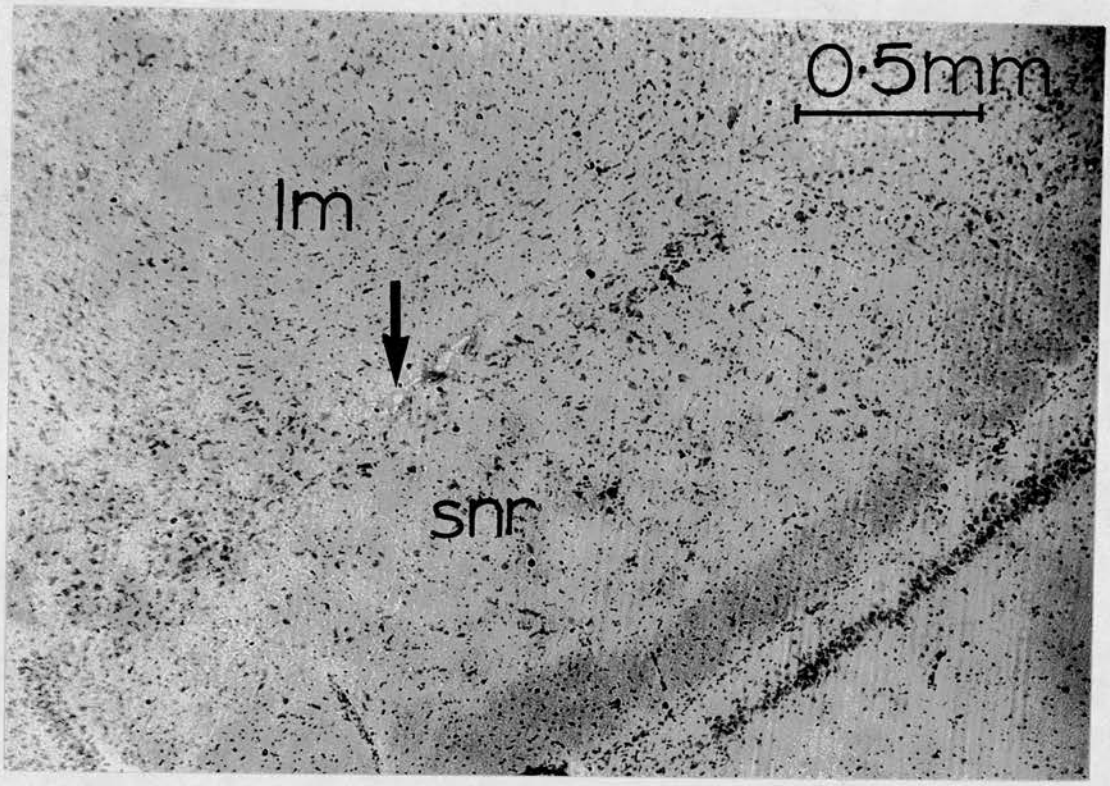

Figure 28

Electrode recording site in the zona compacta region of the substantia nigra. The arrow indicates the lesion produced by the passage of 150 microCoulombs through the tungsten microelectrode, following the single cell recording of a neurone with the firing characteristics of a DA-like cell. The section was stained with cresyl violet and luxol fast blue.

Abbreviations: lm, medial lemniscus; snr, zona reticulata region of the substantia nigra.

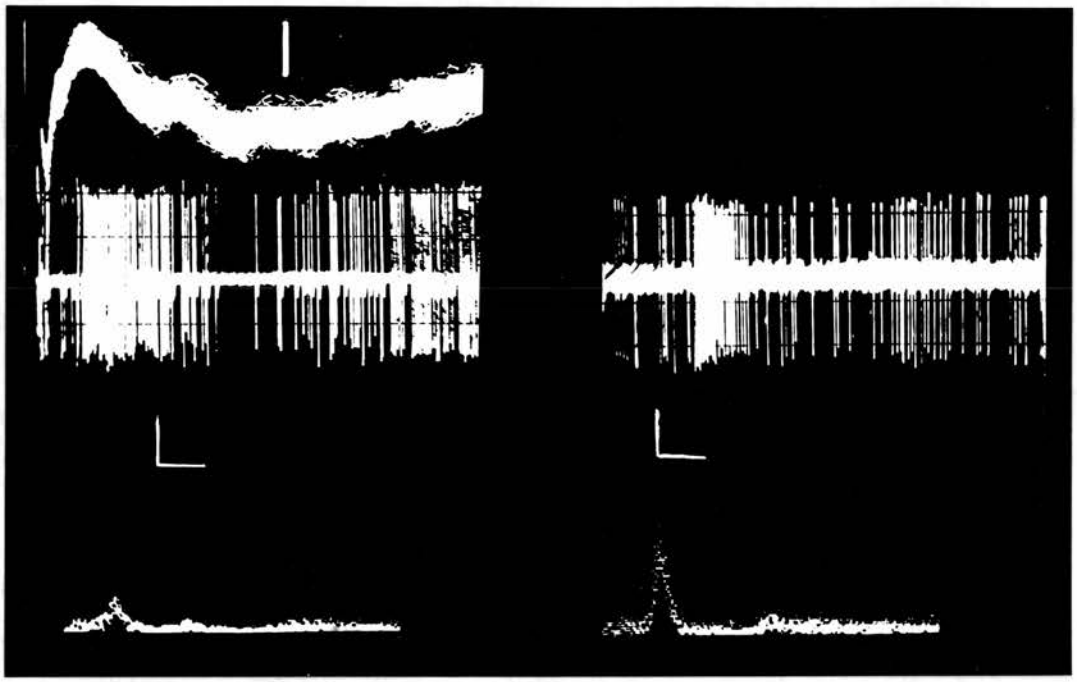

Figure 29

Effect of electrical stimulation of the anterior olfactory nucleus (AON) on the activity of neurones in the ipsilateral zona compacta region. The upper left figure is an evoked slow potential recorded from the contralateral olfactory bulb during stimulation of the AON. The calibration bar is 200μV. The middle figure (left) shows an a.c record (40 superimposed sweeps) of the excitation followed by inhibition of spontaneous neuronal activity after AON stimulation. (Threshold 200μA). Calibration bars 0.25mV (vertical) and 20msec. (horizontal). Below (left) is a computer generated post-stimulus histogram (PSTH) summing the events from the same neurone after 150 consecutive stimuli (sweep duration 160 msec.).

On the right (upper) is an example of a more marked excitation-inhibition response evoked in a different neurone during AON stimulation. Calibration bars 0.1mV (vertical) and 50msec. (horizontal). Threshold stimulation current was 250μA. The PSTH below (sweep 640msec.) represents a summation of events after 150 consecutive stimuli.

All records were initiated by the stimulus.

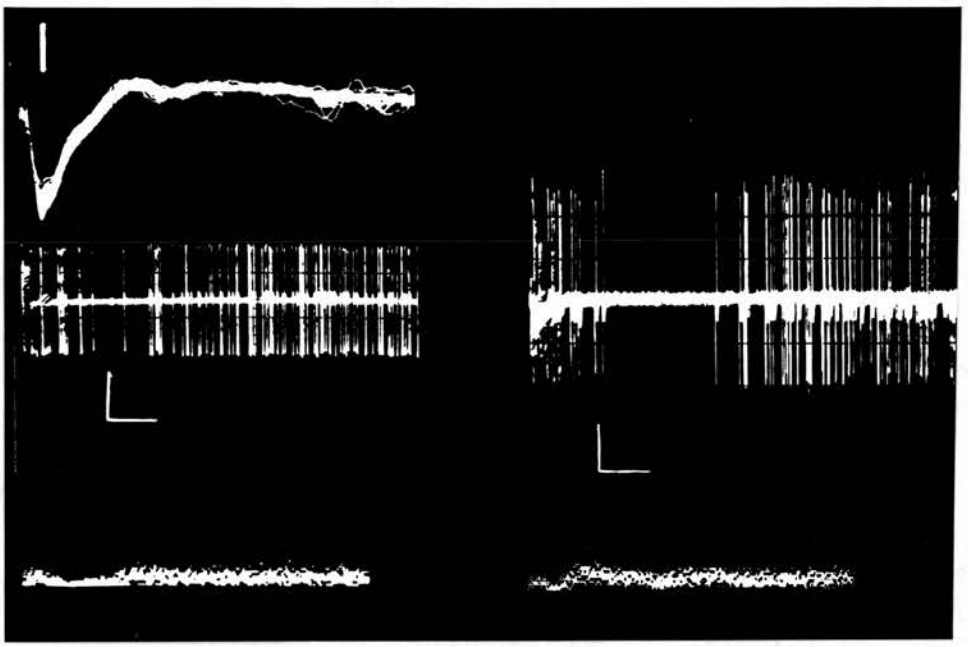

Figure 30

Effects of electrical stimulation of the anterior olfactory nucleus (AON) on the activity of neurones in the ipsilateral zona compacta region. The upper left figure is an evoked slow potential recorded from the contralateral olfactory bulb during a stimulation of the AON. The calibration bar is 0.2mV. The middle figure (left) shows an a.c. record (20 superimposed sweeps) of inhibition of spontaneous neuronal activity after AON stimulatio (Threshold stimulation current 300μA). Calibration bars are 0.5mV (vertical) and 50msec. (horizontal). Below (left) is a computer generated post-stimulus histogram (PSTH) summing the events from the same neurone after 150 consecutive stimuli (sweep duration 640 msec.).

On the right (upper) is an example of a more marked inhibition evoked in a different neurone during AON stimulation. Calibration bars 0.25mV (vertical) and 20msec. (horizontal). Threshold stimulation current was 210 μA. The PSTH below (sweep 640 msecs.) represents a summation of events after 150 consecutive stimuli.

All records were initiated by the stimulus.

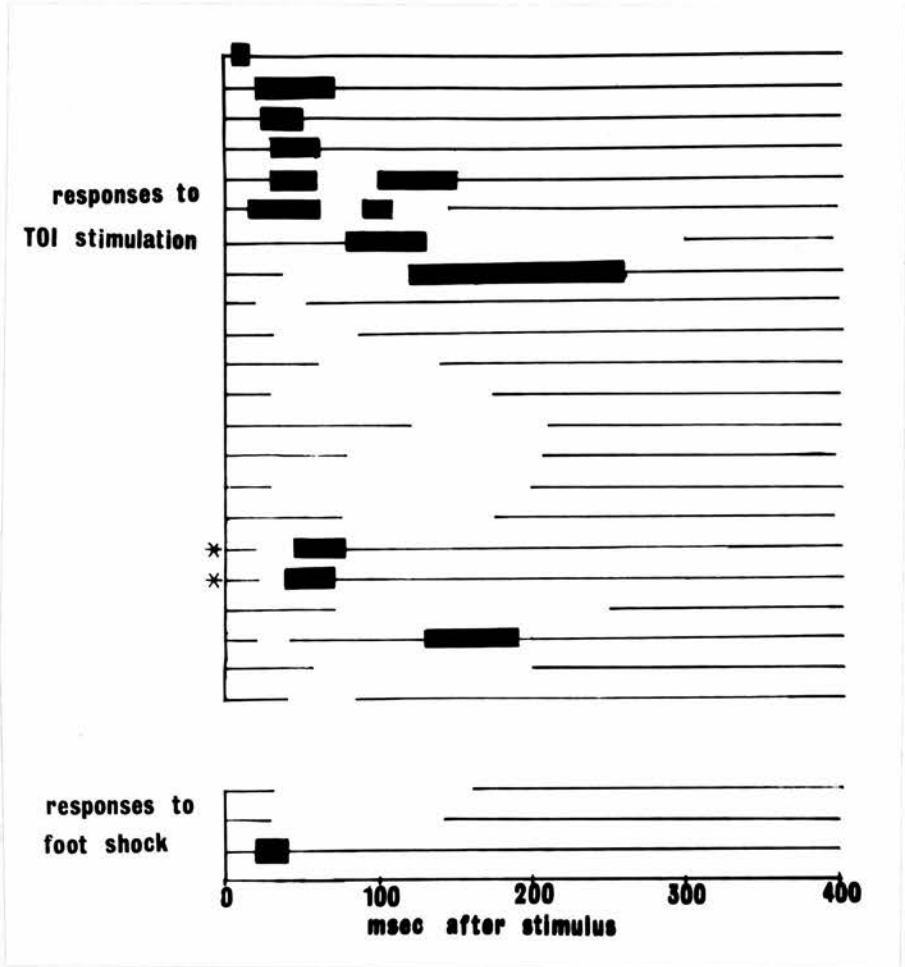

Figure 31

Summary diagram of the responses of neurones in the zona compacta or zona reticulata during electrical stimulation of the anterior olfactory nucleus or foot shock. Each horizontal line represents a 400 msec. post-stimulus period for each responsive cell. A thickening of a line represents an excitation of spontaneous firing rate and a blank indicates an inhibition of firing rate. All responsive neurones, except the two identified zona reticulata neurones (*), were located in the zona compacta and had the firing characteristics of a DA-containing cell.

and the olfactory tubercule directly caudally. The peduncle appears as a trilaminated structure surrounding the olfactory ventricle and consists of three distinct layers. The outer layer predominantly contains main olfactory bulb efferent fibres coursing in the lateral olfactory tract. A middle layer consists of the cells of the AON and the inner layer, which surrounds the olfactory ventricle, contains the AON efferent fibres. The majority of anatomical reports concerning the efferent pathways of the AON have made use of degeneration techniques following lesions of this nucleus (Powell, Cowan and Raisman, 1965; Ferrer, N.G., 1969). This particular approach has the disadvantage that fibres of passage are damaged by the lesion and contribute to the overall degeneration pattern observed. The approach adopted here using the autoradiographic tracing technique is valuable in that labelled leucine is only accumulated by the perikariya of neurones and not by fibres of passage. Thus, the present experiment was designed to map out the efferent pathways from the AON using this technique.

The stimulating electrode placements described in the previous section on the electrical stimulation of this nucleus were all well localised in the inner and middle layers of the olfactory peduncle. In the 6 animals receiving direct AON injections of radioactive leucine the label was well localised with no apparent involvement of the prepyriform cortex, olfactory tubercule or main olfactory bulb. Fig.32 shows an injection site localised in the AON.

In all 6 animals labelled AON efferent fibres could be traced rostrally into the ipsilateral main olfactory bulb. This was unlikely

to be due to spread of label since silver grains were found 3mm. and more rostral to the injection site.

Caudal to the injection site labelled AON axons entered the ipsilateral cerebral hemisphere in the anterior limb of the anterior commissure. Labelled AON axons left the anterior limb in a postero-ventro- lateral direction. Heavy labelling was observed in the region of the claustrum, the polymorph and pyramidal cell layers and the deep half of the plexiform layer of the prepyriform cortex. The olfactory tubercule was also quite heavily labelled. Silver grains above background level were present in the rostral part of the lateral hypothalamic area but it proved impossible to trace this further caudally. Fig.33 shows the grain distribution following the most complete labelling of the AON. All injected brains were found to have a similar pattern of labelling but there was a slight difference in the intensity observed in different areas. In sections caudal to those shown in Fig.33 the only labelling that could be observed was in the ipsilateral pyriform cortex and entorhinal cortex. Not all of the labelled AON efferent fibres within the anterior limb of the commissure left it ipsilaterally. Labelled fibres were clearly seen crossing the midline within the anterior commissure (Fig.34). A large projection left the contralateral anterior limb in an antero-ventro-lateral direction and a concentration of silver grains, above the background level appeared over all the layers of the anterior prepyriform cortex. The labelling continued rostrally into the olfactory peduncle and a concentration of silver grains was present in all regions of the AON. Labelled fibres also entered the main olfactory bulb.

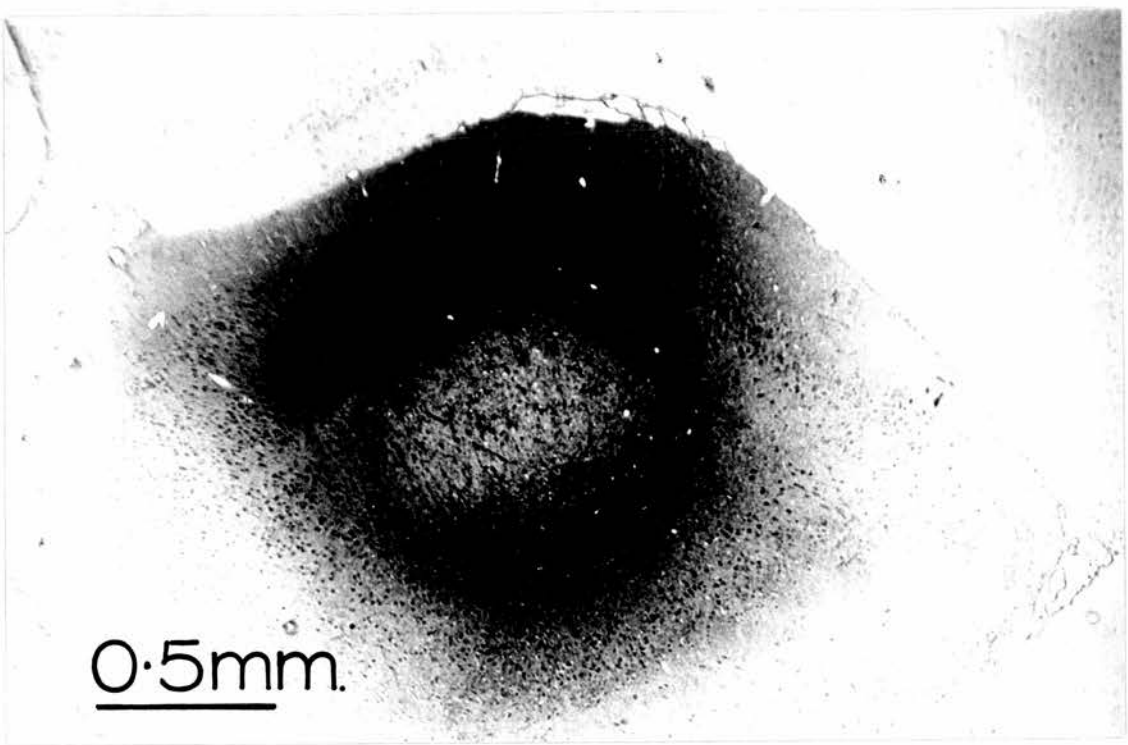

Figure 32

Dense labelling in the anterior olfactory nucleus following an injection of ^{3}H-leucine into this area. Labelled cells can be seen in the peripheral region of the injection site.

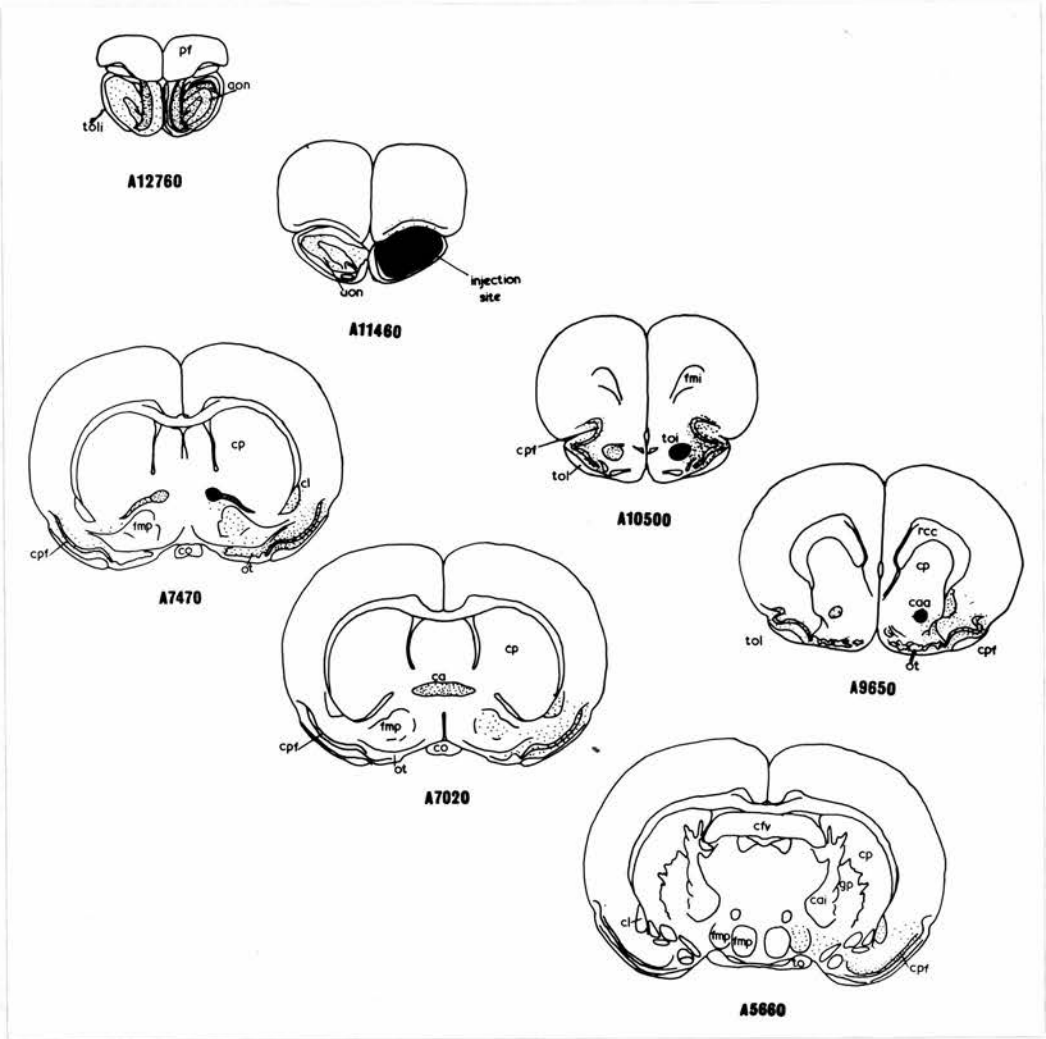

Figure 33

Diagramatic representation of silver grain distribution in rat brain following ^{3}H-leucine injection into the anterior olfactory nucleus. Abbreviations: aon, anterior olfactory nucleus; caa, anterior limb of the anterior commissure; cai, internal capsule; cfv, commissura fornicis ventralis; cl, claustrum; co, optic chiasma; cp, corpus striatum; cpf, prepyriform cortex; fmi, forceps minor; fmp, medial forebrain bundle; gp, globus pallidus; ot, optic tract; pf, polus frontalis; tol, lateral olfactory tract; tOli, tractus olfactorius lateralis, pars intermedia.

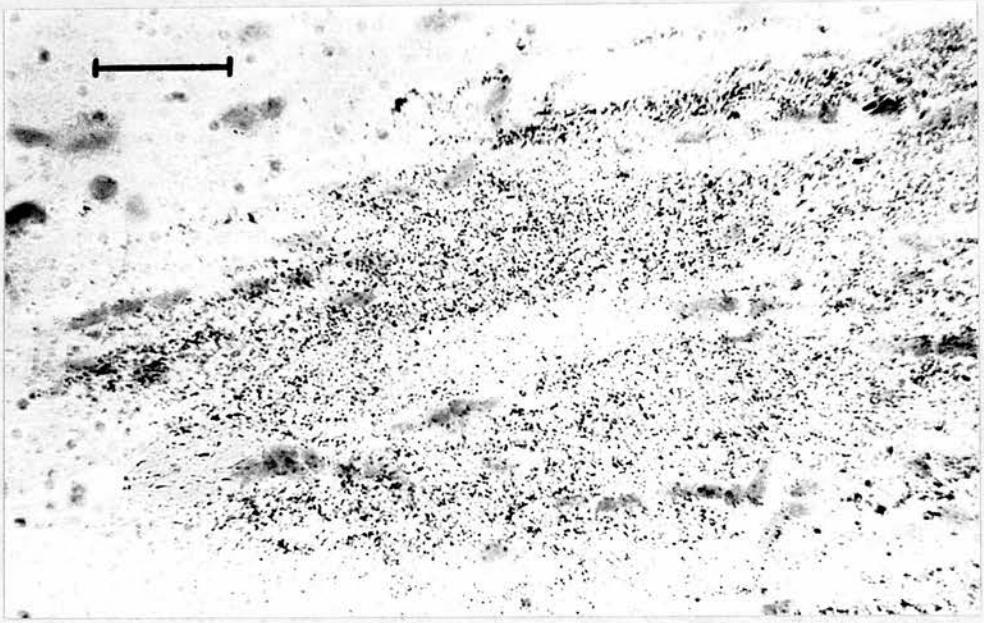

Figure 34

Presence of silver grains in the anterior commissure following injection of ^{3}H-leucine into the anterior olfactory nucleus (shown in Fig.32). Scale bar; 25μm.

Efferent Connection of the Lateral Hypothalamus

In order to establish whether path neurones located in the medial forebrain bundle projected to the substantia nigra it was thought worthwhile to do some preliminary tracing experiments similar to those described for the AON. The majority of previous experiments on the efferent pathways of the lateral hypothalamus suffer from the same disadvantage as that outlined for the AON i.e. they all involved lesions of this area and hence must have caused a considerable amount of damage to fibres of passage (Guillery, R.W., 1957; Nauta, W.J.H., 1958; Wolf and Sutin, 1965). The present experiment was an attempt to overcome these limitations and provide evidence for a possible lateral hypothalamic- substantia nigra projection that had its origin in cells located in the medial forebrain bundle.

All injections of labelled leucine were placed in the region of the lateral hypothalamus but considerable difficulty was encountered in achieving good localisation of the label. Only in 5 out of the 12 injected rats was the label well localised and concentrated over the lateral hypothalamic area with only a minimal spread to the surrounding areas. All the other injected animals were discarded from this study due either to the autoradiographic material having too high a background count or more commonly because of a poorly localised injection site.

The 5 successfully injected animals all had label localised over the region of the lateral hypothalamus. The typical labelling pattern at the site of injection was an intense concentration of grains within cells in the area and a very high concentration of

grains located in the extracellular region surrounding these cells. An injection site from one of these animals is shown in Fig.35. The course and distribution of labelling observed in the brains with the successful injections were qualitatively similar and only one will be described here. The silver grain distribution present in different planes of brain section following the injection illustrated in Fig.35 is shown in Fig.36.

Labelled axons ascending from the injection site were followed through the medial forebrain bundle and two main fibre tracts could be clearly seen coursing in a dorsal direction. At the level of the anterior commissure labelled axons were seen to course towards the septal region and quite heavy labelling was observed in the lateral septal region (Fig.37(a)) with the medial septum only being lightly labelled. Dorsally directed fibres were also seen at the level of the anterior hypothalmus. In this instance the majority of fibres entered the stria medullaris (Fig.37(b)) but it proved difficult to follow the exact pathway taken by these fibres. The labelled fibres within the stria medullaris were traced in a caudal direction to the habenular nucleus, where heavy labelling was observed in the lateral region (Fig.37(c)). Note the absence of labelling in the contra-lateral lateral habenular nucleus (Fig.37(d)).

Caudal to the injection site heavy labelling was seen in the medial forebrain bundle as far back as the mammillary bodies. At this level there was a clear divergence of labelled fibres heading in a dorsal direction. A medial pathway projected to the ipsilateral region of the central gray and terminated there. A second pathway,

also arising from the medial forebrain bundle projected in a lateral-dorsal direction and could be followed to the mesencephalic reticular formation. This pathway coursed through the zona compacta region and the area immediately dorsal to that. Fig.38 shows the pattern of labelling that was observed in the zona compacta region. From the data presented here it is clearly impossible to establish whether the labelling in this region represented terminals or fibres of passage. Caudal to the substantia nigra there was only light labelling present in the ventral region of the brain. The highest labelling was found in the central gray region. A few dorsally directed labelled fibres were found in the posterior midbrain and they seemed to merge into the labelling located in the central gray region. Sections caudal to A350 from the atlas of König and Klippel were not studied in this present investigation.

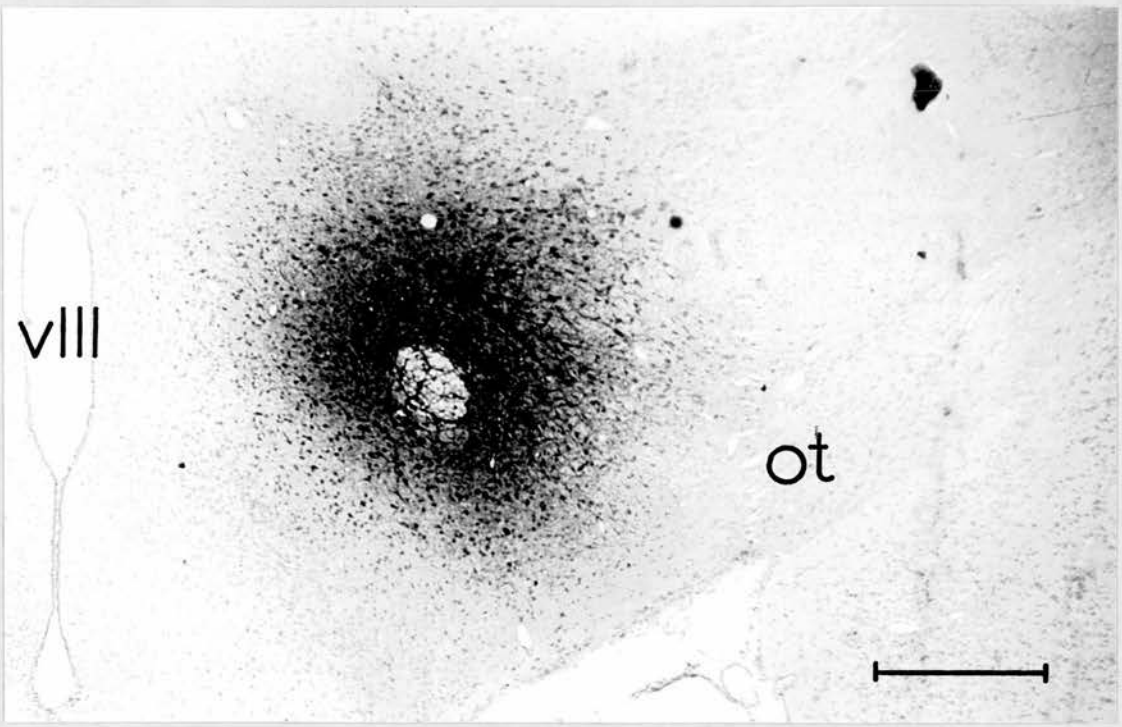

Figure 35

Injection site of ^{3}H-leucine into the lateral hypothalamus. This photomicrograph was taken from an autoradiograph that had been exposed for 30 days at -20°C. Scale bar; 0.5mm.

Abbreviations: ot, optic tract; vIII, third ventricle

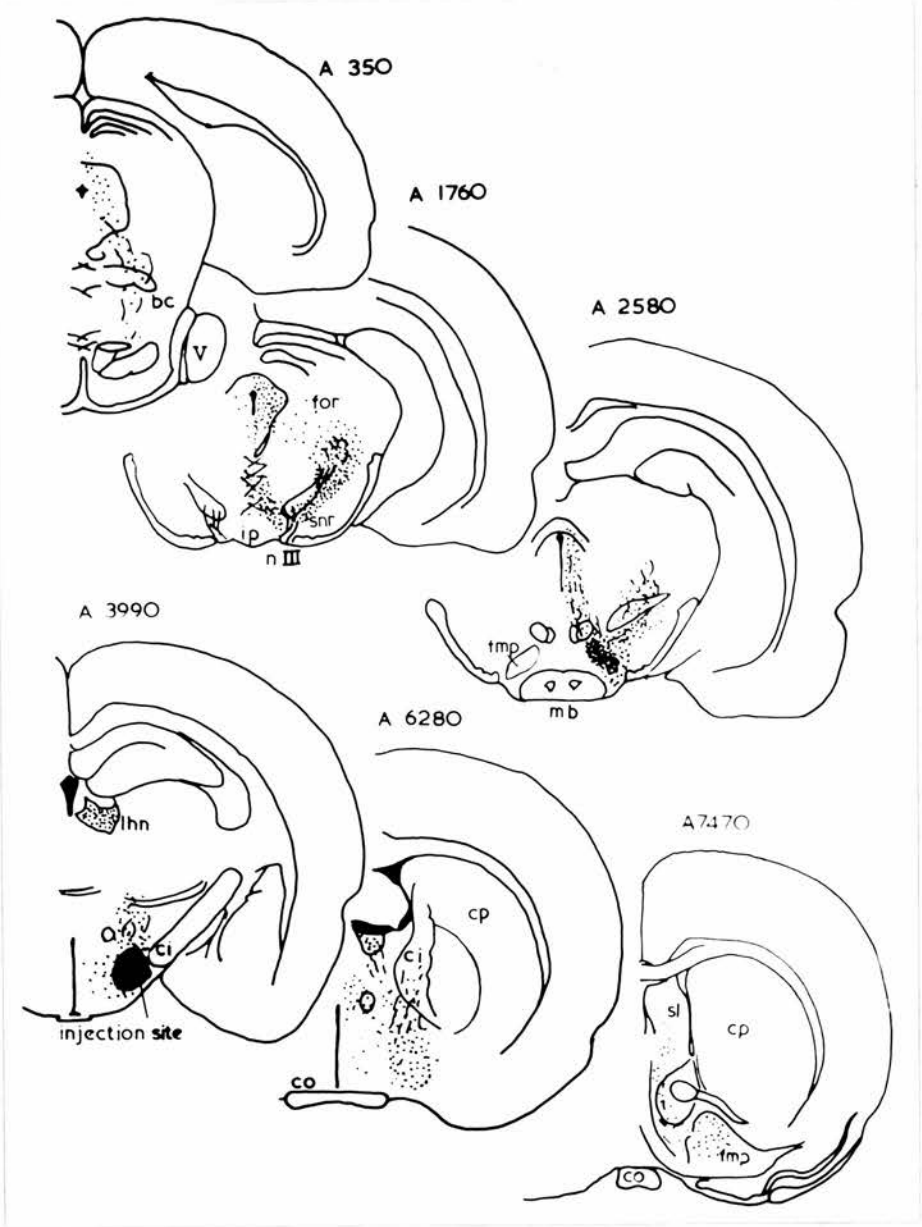

Figure 36

Schematic diagram showing the distribution of labelled axons and terminals in brain following an injection of ^{3}H-leucine into the region of the lateral hypothalamus. The post-injection survival time was 8 hours.

Abbreviations: bc, brachium conjunctivum; for, midbrain reticular formation; fmp, medial forebrain bundle; ci, internal capsule; cp, corpus striatum; lhn, lateral habenular nucleus; sl, lateral septum; co, optic chiasma; snr, substantia nigra, zona reticulata region; ipn, interpeduncular nucleus; mb, mammillary bodies; V, fifth cranial nerve

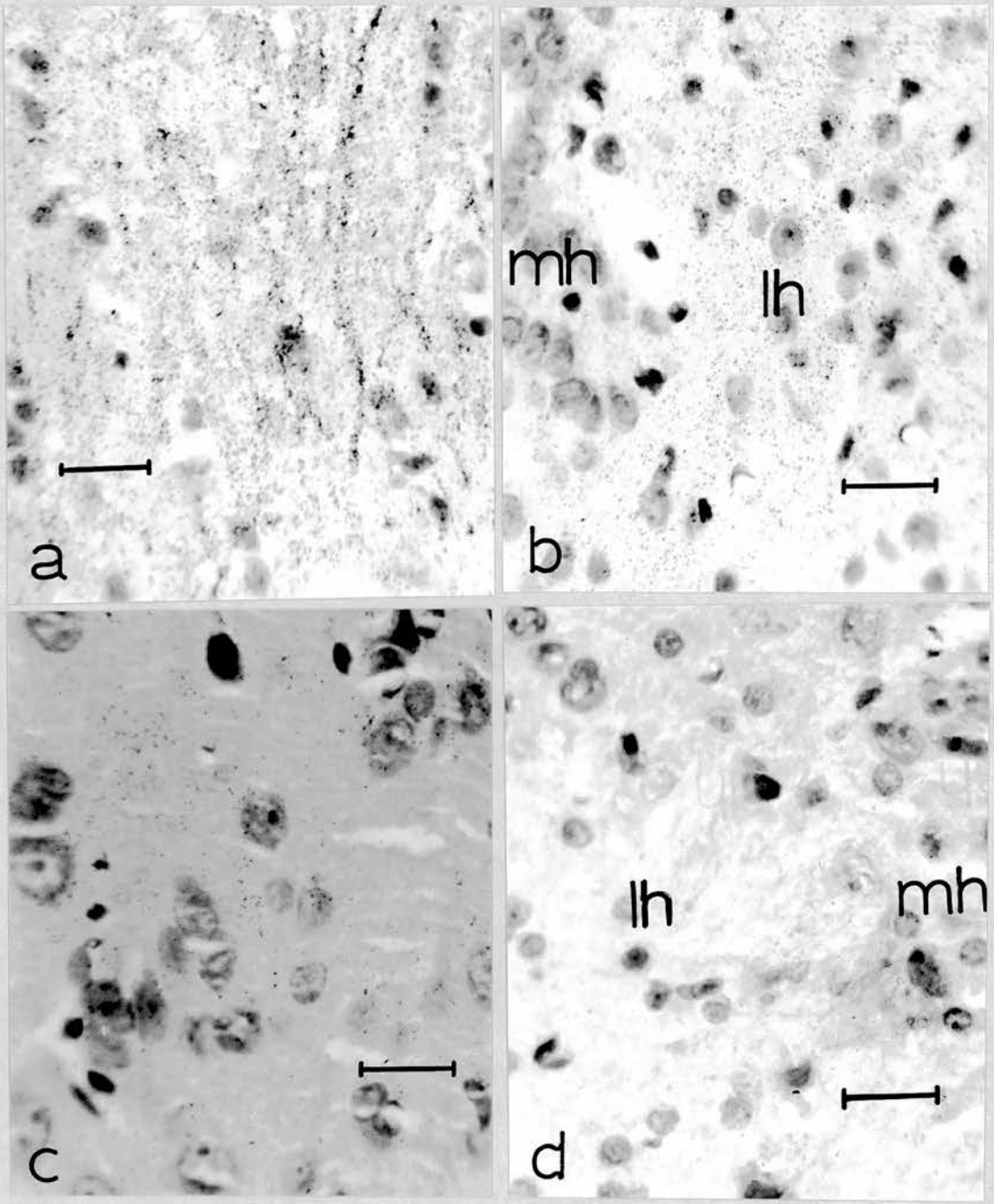

Figure 37(a)

Presence of silver grains in axons about to enter the stria medullaris following an injection of ^{3}H-leucine into the region of the lateral hypothalamus (injection site shown in Fig.35)

Figure 37(b)

Presence of silver grains in the ipsilateral habenular nucleus (lh) in the same animal. Note the absence of grains in the medial habenular nucleus (mh).

Figure 37(c)

Presence of silver grains in the ipsilateral lateral septal region.

Figure 37(d)

Absence of silver grains in both the medial and lateral habenular nuclei on the non-injected side. Scale bar; 20 μm.

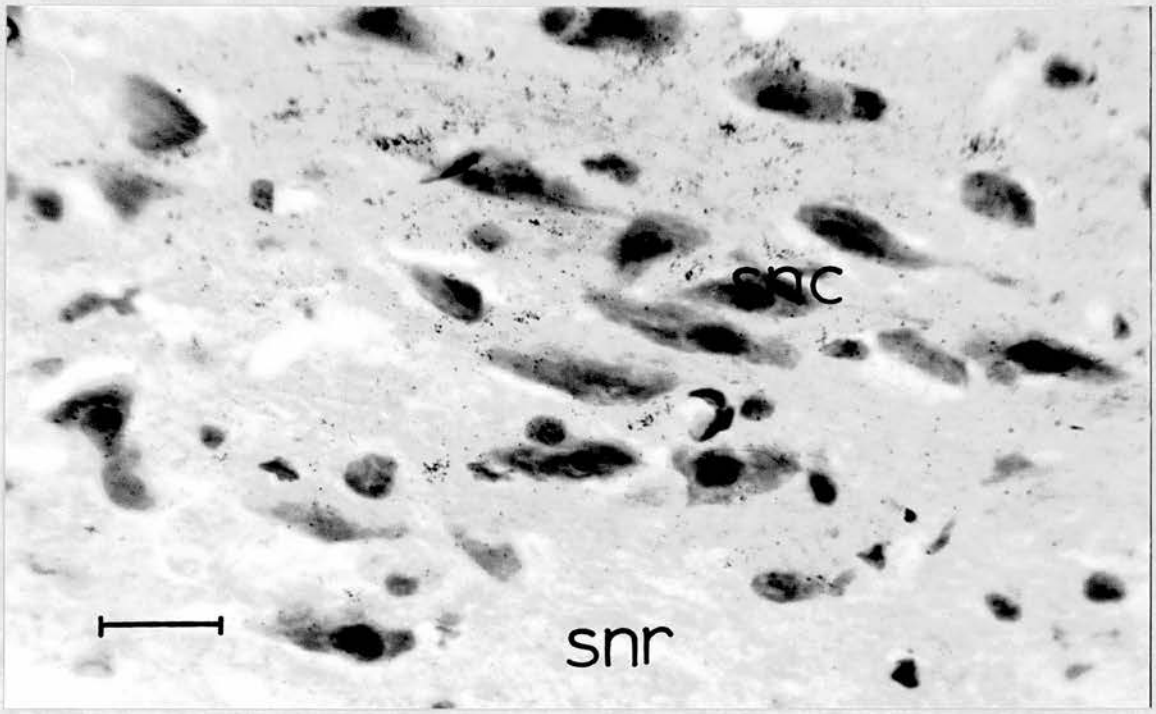

Figure 38

Distribution of silver grains in the substantia nigra of an animal injected with ^{3}H-leucine in the region of the lateral hypothalamus (injection site shown in Fig.35). The grains are localised to the zona compacta (snc) region and the area immediately dorsal to it. Note the absence of grains in the zona reticulata region (snr). Scale bar; 20 μm.

DISCUSSION

In the present section of work an attempt has been made to define, using both neuroanatomical and electrophysiological techniques, possible afferent pathways to the DA-containing cells in the substantia nigra. As pointed out previously, there is an obvious lack of anatomical evidence available on which to base possible electrophysiological experiments and this study is only a modest beginning since it does not establish direct projections to the zona compacta region. The recently described retrograde neuroanatomical mapping technique utilising horseradish peroxidase enzyme (La Vail and La Vail, 1972) would probably have proved to be a better initial investigative method since it would have provided clues about which brain areas project directly to the SN zona compacta region. This technique was employed in the next section of work. Therefore, since the temporal order of the experiments was such that the electrophysiology and autoradiography experiments were performed prior to the horseradish peroxidase experiments it is pertinent to discuss the results of the former here.

Throughout the course of the present study the central idea tested was whether or not olfactory information influenced DA cell activity. Crow, 1973, stressed that the habenulo-interpeduncular pathway could possibly be important in relaying olfactory information to the DA-containing cells in the substantia nigra. Few anatomical studies, concerning this pathway, have been reported in the rat (Lenn, 1976) although it is well documented in other mammalian species, including the opossum (Smaha and Kaelber, 1973),

cat (Akagi and Powell, 1968; Smaha and Kaelber, 1973) and rabbit (Mizuno and Nakamura, 1974). All these reports are in good agreement with the findings in the present study. The major efferent output from the lateral and medial habenular nucleus projects massively to the interpeduncular nucleus via the fasciculus retroflexus of Meynart. This projection has also been confirmed electrophysiologically (Lake, 1972) where it is reported that it exerts a predominantly excitatory effect on interpeduncular neurones. Smaha and Kaelber, 1973, reported fibre degeneration that was dorsal to the interpeduncular nucleus and were able to trace this pathway to its termination in the tegmental nuclei of Gudden. The sections studied here did not extend sufficiently far caudal to verify these findings but it is likely that the degeneration observed in the medial raphe region was fibre degeneration of this caudal projection of the habenular nucleus, since it is co-extensive with that reported by Smaha and Kaelber. Certainly, very little sign of degeneration was found in the zona compacta region but it did appear to be present in an area immediately dorso-medial to the interpeduncular nucleus. This was probably axonal in origin and constituted the degenerating caudal projection of the habenular nucleus to the tegmental nuclei. Even although silver impregnation techniques are notorious for false positive and negative results (Heimer, 1972) there does appear to be firm agreement that the interpeduncular nucleus is the principal terminal area of the habenulo- interpeduncular tract.

The electrophysiological experiments, performed to investigate the possibility of an indirect projection from the habenular nucleus to the DA neurones, showed that neurones located throughout the zona

compacta, ventral tegmental area and zona reticulata were singularly unresponsive to electrical stimulation of both the lateral and medial habenular nuclei. Thus, although it is still possible that olfactory information could influence DA cellular activity in the SN it would appear that the habenular nucleus is not involved in relaying such information to the DA cells.

Electrical stimulation of the olfactory bulb has often been employed in studies on olfactory pathways in the central nervous system (Scott, J.W., and Pfaffmann, C., 1967; Pfaff and Pfaffmann, 1969; Komisaruk and Beyer, 1972; Motokizawa, 1974). From the reported literature it is difficult to establish the sites of olfactory bulb stimulation since they are not usually reported. However, in one recent study, Mok and Mogenson, 1974, reported that electrical stimulation of the olfactory bulb, in the region of the mitral cell layer, elicited responses from lateral habenular neurones. The stimulation voltages used were in the range 3-12 V, employing both single pulse and train stimulation. In the present study the stimulation parameters and sites were largely based on those described by Mok and Mogenson. In both studies the stimulation sites were usually close to the mitral cells of the bulb. These cells are known to be the main output cells of the olfactory bulb and it is their axons which gather to form the lateral olfactory tract (MacLeod, 1972). The stimulation current used in the present study, 50-200uA corresponding to a voltage range of 4-20 V should have been adequate to stimulate the output pathway of the main olfactory bulb. Although the number of zona compacta neurones studied was small (n=7), none of them were found to be influenced by olfactory bulb stimulation.

The lateral olfactory tract is the only direct projection of the olfactory bulb to the forebrain (Powell et al., 1965). During their course, the fibres in this bundle emit numerous collaterals, making direct ipsilateral connections with all the subdivisions of the anterior olfactory nucleus (Scalia, 1966). From lesion studies it is known that the anterior olfactory nucleus projections overlap considerably with the lateral olfactory tract projections to the prepyriform and pyriform cortices and olfactory tubercule (Powell et al., 1965; Ferrer, 1969; Broadwell, 1976). Thus, electrical stimulation of this nucleus would be expected to influence all these primary olfactory areas. The finding that electrical stimulation of the AON elicited marked and complex responses from a high percentage of zona compacta neurones (70%) was in stark contrast to that observed following olfactory bulb stimulation. Perhaps the most likely explanation of these puzzling results was that a varying amount of damage to the olfactory bulb occurred during the exposure and preparation of this structure for stimulation. Therefore a question mark must be placed against the functional integrity of the stimulated olfactory bulb. In contrast, it was possible, in each experiment involving AON stimulation, to check that the electrical stimulation was activating the AON output fibres. From previous anatomical findings it was known that the AON projected to the contralateral olfactory bulb (Powell et al., 1965; Scalia, 1966) and in the present experiments it was possible to record an evoked potential from the bulb following stimulation of the AON. This large evoked potential served to check the position of the stimulating electrode, prior to recording, as well as the successful stimulation of the AON output pathway.

The fact that halothane anaesthesia was used may also have had an important effect on the results. Many workers, employing olfactory bulb stimulation have used urethane anaesthesia (Scott and Pfaffmann, 1967, 1972; Pfaff and Pfaffmann, 1969; Scott and Leonard, 1971; Mok and Mogenson, 1974). The author knows of no olfactory experiments that have used halothane anaesthesia but it has been reported that olfactory bulb stimulation can elicit responses from lateral hypothalamic neurones under a wide range of anaesthetic conditions (Scott and Pfaffmann, 1972). Thus, it is possible that the stimulation currents used in the bulb experiment were insufficient, under halothane anaesthesia, to activate the output fibres. It is obvious that the stimulation currents required to elicit responses from DA neurones by AON stimulation were generally greater than those used to stimulate the olfactory bulb.

The latencies of the DA cell responses to AON stimulation are not unexceptional when compared with many other olfactory studies concerning neuronal responses to electrical stimulation of the olfactory bulb. For example, Mok and Mogenson, 1974, reported that olfactory bulb stimulation elicited both facilitatory and inhibitory responses from lateral habenular neurones, with latencies ranging from 12-180 msecs. From anatomical evidence (Powell et al., 1965) it is evident that the primary olfactory cortex projects directly to the lateral habenular nucleus, thus, it is possible that this nucleus is functionally separated from the olfactory bulb by only two synapses. Mok and Mogenson concluded from their results, not surprisingly, that the lateral habenular nucleus did not receive direct connections from the olfactory bulb. Other olfactory studies,

in the lateral hypothalamic region have also reported a considerable range in the latency of neuronal response. Scott and Pfaffmann, 1972, reported that the lateral hypothalamus neurones could be driven by olfactory bulb stimulation with latencies in the range 3-90 msecs. Therefore, it seems that direct pathways, involving two or three synapses, may be involved in mediating these effects, along with more diffuse, polysynaptic pathways. From the response latencies in the present study it is apparent that the pathway involved is polysynaptic, since the majority of cells responded within 20-40 msecs. after the stimulus.

Many workers have used direct odour stimulation to further test central neurones for specific responsiveness to olfactory stimulation. The overwhelming problem with this approach, as well as the electrical stimulation method, is the possibility of non-specific arousal (Pfaff and Pfaffmann, 1969; Komisaruk and Beyer, 1972). The evidence that olfactory stimulation does have an arousal effect in the brain is principally based on EEG arousal elicited in the neocortex and hippocampus by olfactory bulb and odour stimulation (Arduinni and Moruzzi, 1953; Motokizawa, 1973). In the high cerveau isole cat preparation, sectioned at the mesencephalic-diencephalic junction, it has been shown that this arousal effect of olfactory stimulation is abolished (Motokizawa and Furuya, 1973). Therefore, these workers suggested that removal of the mesencephalic reticular formation, an area in which individual neurones are known to be responsive to more than one sensory modality (Amassian and De Vito, 1954; Machne, Calma and Magoun, 1955; Schiebel, Schiebel, Mollica and Morruzi, 1955; Bell, Sierra, Buendia and Segundo, 1964) resulted in the abolition of

the arousal effect. Single cell studies in the cat (Motokizawa and Furuya, 1973) showed that olfactory bulb stimulation had a pronounced effect on mesencephalic reticular formation neurones. They found that by completely lesioning the medial forebrain bundle it was possible to block this effect. It was also observed that the neocortical arousal response disappeared following these lesions. Therefore, it was proposed that the medial forebrain bundle relayed olfactory information to neurones in the reticular formation but that the function of this was to alert the animal. In the present study it would have been helpful if similar medial forebrain bundle lesions could have been performed. However, the axons of the nigro-striatal and mesolimbic DA systems course through this bundle and so the possibility of damage to these axons by such lesions precluded such an experiment.

Another useful approach used in olfactory studies is to record neuronal responses to odours whilst recording cortical EEG and various peripheral parameters (Pfaff and Pfaffmann, 1969). If it can be shown that neuronal responses can occur independently of arousal changes this is taken as providing good evidence for a responsiveness specifically to olfactory stimulation. With the odours in common usage in olfactory studies e.g. xylene it is surprising that arousal responses are not elicited by all such novel compounds.

The method most usually employed to establish a non-specific mode of stimulation is to test the responsiveness of neurones to an array of non-olfactory stimuli e.g. foot shock or tail pinch, auditory clicks or flashing lights (Pfaff and Pfaffmann, 1969; Scott

and Pfaffmann, 1972; Komisaruk and Beyer, 1972; Motokizawa, 1974). The use of foot shock in the present study was sufficient to cause an obvious foot withdrawal and could be considered highly arousing. The fact that such a low percentage of zona compacta neurones responded to this form of stimulation suggests that the DA neurones may be responsive to a specific olfactory input. However, since other tests for non-specific responses were not performed it cannot be stated whether these forms of stimulation would elicit responses in DA neurones. The latencies of the peripheral responses were similar to those of the "olfactory" responses.

It is now quite well established from anatomical studies, that the primary olfactory areas project to the lateral hypothalamus via the medial forebrain bundle. (Powell et al., 1965; Scott and Leonard, 1971; Heimer, 1972; Scott and Chafin, 1975). Neurones, located in the lateral hypothalamus are known to be responsive to olfactory stimulation (Barraclough and Cross, 1963; Scott and Pfaffmann, 1967; 1972; Pfaff and Pfaffmann, 1969; Scott and Leonard, 1971). Millhouse, 1969, using the Golgi staining technique, demonstrated that the medial forebrain bundle was an exceptionally complex pathway involving both ascending and descending components. This worker suggested that the ventral portion of the descending MFB was a collection of axons originating in the AON, olfactory tubercule amygdala and the pre-pyriform cortex which course caudally through the lateral hypothalamus into the rostral mesencephalon. The anatomical data presented in this section does suggest that the AON does project to the rostral hypothalamus but certainly it did not project to the mesencephalon. However, it is quite conceivable that there is a functional connection

between these areas since the path neurones within the MFB have contacts with many MFB fibres and these path neurones, in turn, project in both caudal and rostral directions (Millhouse, 1969). At the junction of the mesencephalon and the diencephalon, Millhouse reported that the MFB fans out into the tegmentum and that the fibres had numerous boutons en passant and short collaterals synapsing with tegmental reticular neurones.

If one has to invoke a pathway by which AON stimulation elicited responses in DA neurones then the MFB looks the most likely candidate. To further investigate the problem of a possible MFB projection to the nigral DA system and mesencephalic reticular formation the autoradiographic tracing technique was used. Previous reports on the efferent connections of the medial forebrain bundle, with one exception (Troiano and Siegel, 1975) had all employed lesion studies and silver impregnation methods (Guillery, 1957; Nauta, 1958; Wolf and Sutin, 1966), therefore the results must be interpreted with the possibility that fibres of passage in the MFB contributed to the observed degeneration pattern. The results from the present work, on the efferent projections of path neurones in the MFB do show remarkable agreement with these previous studies. The pathway of particular interest was the caudally-directed projection which coursed through the dorsal region of the substantia nigra and seemed to terminate in the reticular formation. Nauta reported from his lesions studies in the cat that "Immediately caudal to the lesion, the MFB appears as the central structure in a widespread field of fibre degeneration. It occupies its characteristic position ventral to the red nucleus. Spreading from the bundle in a laterodorsal direction are degenerating

slender fascicles which distribute to the tegmental region adjoining the SN on the dorsal side". From Fig.38 it can be clearly seen that the slender fascicles do course through the zona compacta region but it is uncertain whether or not they synapse with the DA neurones.

Electrolytic lesions placed in the zona compacta region have been reported to produce retrograde chromatolysis in neurones in the lateral hypothalamus (Routtenberg, 1974). This result does not add further support to the existence of a lateral hypothalamic- zona compacta pathway since these lesions would have interrupted the efferent fibres of the lateral hypothalamus neurones as they coursed through the zona compacta. From the previously reported results and from those obtained in the present tracing study it cannot be stated with certainty that lateral hypothalamic neurones project to the zona compacta neurones.

It is known that sites in the lateral hypothalamus support the highest rates of self-stimulation in the brain, and it has been suggested that stimulation of the ascending monoamine fibres coursing through this area may be important in this behaviour (Dresse, 1966). However, it is very difficult to separate out the effects of catecholamine fibre stimulation from those following stimulation of the efferent fibres of the lateral hypothalamus since these fibre systems are co-extensive in the medial forebrain bundle. More precise catecholamine lesion studies on self-stimulation behaviour are required before this problem can be resolved.

In summary, evidence is presented here for the following

conclusions:

(a) The habenular nucleus does not project directly to the zona compacta region of the SN since no significant terminal degeneration could be detected in that region following ablation of the habenular nucleus.

(b) DA-containing neurones in the SN are not influenced by electrical stimulation of the medial and lateral habenular nuclei.

(c) Electrical stimulation of the ipsilateral olfactory bulb failed to influence the firing rates of neurones located in the substantia nigra.

(d) A high percentage (70%) of DA-containing neurones in the SN respond in a complex and variable manner to relatively high intensity stimulation of the ipsilateral anterior olfactory nucleus. The possibility of this being a non-specific effect is not ruled out.

(e) Neurones in the lateral hypothalamus project mainly to the lateral septal area, lateral habenular nucleus, midbrain reticular formation and the periventricular grey. Some efferent fibres from these neurones course through the zona compacta region of the SN but no evidence is forwarded to establish whether or not these fibres terminate in this region.

CHAPTER IV

AN INVESTIGATION INTO THE AFFERENT PATHWAYS PROJECTING TO THE SUBSTANTIA NIGRA UTILISING THE HORSERADISH PEROXIDASE RETROGRADE TRACING TECHNIQUE

INTRODUCTION

The inherent disadvantages of the anterograde neuroanatomical tracing techniques as used at the resolution of the light microscope were highlighted in the previous chapter. Apart from the obvious difficulty in distinguishing between labelled or degenerating terminals and fibres of passage it is also apparent that a certain foreknowledge of the anatomical projections to the neuronal system under investigation is required before these techniques can be used other than on a trial and error basis. Until recently the only retrograde method available whereby the cellular origin of axon terminal networks could be traced were the cell degeneration techniques based on the work of Gudden (1870) and of Nissl (1892). These depend on the chromatolytic changes occurring in the cell bodies following axotomy. Although they have been widely used these techniques suffer from a number of serious limitations; in particular the difficulty encountered in establishing whether the observed chromatolysis is due to retrograde, anterograde or retrograde transsynaptic cell degeneration (Powell and Cowan, 1967; La Vail 1975).

The finding that peripheral nerves could retrogradely transport labelled amino acids (Kerkut, Shapira and Walker, 1967; Watson 1968) was highly significant in the development of a better retrograde neuroanatomical technique. Following these findings it was soon established that proteins, including the enzyme horseradish peroxidase (HRP) could also be retrogradely transported along motor fibres of the sciatic nerve (Kristensson and Olsson, 1971) and

hypoglossal nerve (Kristensson and Olsson, 1971; Kristensson, Olsson and Sjöstrand, 1971). These workers found that the enzyme was taken up by undamaged nerve endings and transported retrogradely, within the axons, to the cell bodies of origin, where it could be visualised histochemically by the diaminobenzidine (DAB) method described by Graham and Karnovsky, 1966. The technique, as applied to the CNS, was first described in a study on the visual system of the chick. (La Vail and La Vail, 1972). More detailed studies have confirmed that this retrograde transport of HRP is potentially a very powerful technique in the field of neuroanatomy (La Vail, Winston and Tish, 1973; Nauta, Pritz and Lasek, 1974). Since these initial reports appeared there has been a burgeoning of the application of the technique in studies on neural connectivity in the CNS at both the light and electron microscope level. Although there are limitations in defining the precise extent of the effective site of injection as well as certain difficulties in the interpretation of possible artifacts that occur it is apparent that retrograde transsynaptic transfer of HRP and anterograde movement of HRP do not present complicating factors at the light microscope level (La Vail, 1975). The great advantage with the technique is that it is possible to positively identify labelled neurones that project to a given injection site. This fact makes the technique, applied either by itself or in combination with other histochemical methods (Ljungdahl, Hökfelt, Goldstein and Park, 1975), very appealing to the neuro-anatomist.

Recently the HRP technique has been used successfully in demonstrating the striato-nigral pathway in the cat, following

microinjections of the enzyme into the zona reticulata region (Grofova, 1975). However, in the rat, it is noticeable that research reports are still lacking on the afferent connections of the SN using this retrograde technique. The relatively small size of the structure and the difficulty in making well-localised injections are possible explanations for this lack. In spite of these difficulties it was decided in the present study that the technique offered the only possibility of mapping out the neural connections to the zona compacta region of the SN.

Since the autoradiographic studies in Chapter III suggested a possible projection from the lateral hypothalamus to the zona compacta region it was particularly important to establish whether in fact this pathway did terminate in the region of the SN. The study also describes in detail the distribution of retrogradely labelled neurones following HRP microinjections into different parts of the SN. Since initial interpretative difficulties were encountered in the identification of labelled neurones a description of possible confusing artifacts is also presented.

METHODS

Experimental Procedures for the Horseradish Peroxidase Technique

Throughout this study adult, male, albino rats, weighing 190-210 gm. were used. The rats were anaesthetised with a halothane/air mixture (1%) and fixed in a stereotaxic frame (David Kopf Instrument). All HRP injections were accomplished manually by means of a stereotaxically guided 30 gauge needle (Pharmaceutical Mfg. Corporation) connected to a 1µl. syringe (Hamilton).

Initially, 2 rats were injected in the region of the corpus striatum with 0.5µl. of a freshly-prepared 10% solution of HRP (1mg. of HRP-(Boehringer Grade I) was dissolved in 10µl. of 0.1M phosphate buffer, pH 7.4). The injections were performed over a period of 5 minutes and the needle was left in the brain for a further 30 minutes. The purpose of these injections was to check that the adopted HRP experimental protocol, to be described, was in fact working and giving results similar to those described recently by Nauta, Pritz and Lasek, 1974.

16 further rats were injected with HRP in the region of the SN. It was found by trial and error that delivery of 0.15-0.2 µl. of a 10% HRP solution, over a period of 5 minutes resulted in adequate and reasonably well-localised staining of the SN. The injection needle was inserted into the brain at an angle of 45° and the stereotaxic coordinates used were as follows:

Posterior	4.7 mm.
Lateral	6.5-7mm.
Vertical	6.8-7.2mm.

The bregma suture was used as the stereotaxic reference point, except in the case of the vertical coordinate, where the reading was taken from the overlying cortical surface. During the injections aimed for the zona compacta region (lateral coordinate 6.5mm.) the needle was retracted 0.7mm. from the site of most ventral penetration (7.2mm.) in an attempt to confine the HRP to a narrow strip lying along this brain area. After each injection the needle was left in the target area for a further 30 minutes.

All the operated rats had a post-injection survival time of 24 hours and were then reanaesthetised with chloral hydrate (400 mg./kg.) and perfused transcardially with 80 ml. of ice-cold 0.1M phosphate buffer (pH 7.4) followed by 150 ml. of the same 0.1M phosphate buffer containing 2% paraformaldehyde and 2.5% glutaraldehyde. The entire rat brain was removed immediately after the perfusion and placed in the same fixative for a further 24 hours. Finally the fixed brain was transferred to 0.1M phosphate buffer (pH 7.4) containing 5% sucrose and kept there for a further 24 hours.

The brains were cut coronally into two approximately equal parts and each mounted on a piece of cork which was then frozen onto a cryostat chuck. Sections 40 µm. thick were collected serially in different compartments of 27-compartment plastic ice-boxes containing ice-cold 0.1M Tris-HCl buffer (pH 7.6). All the sections were rinsed

in this buffer for at least 2 hours and no more than 4 hours. It has recently been reported that during this time required for washing diffusion of the enzyme may occur if the sections are washed for long periods (La Vail, 1975). The sections were then presoaked for 30 minutes at room temperature in a freshly-prepared solution containing 50 mg. 3-3 - diaminobenzidine tetrahydrochloride (DAB - Sigma) in every 100 ml. of 0.1M Tris-HCl buffer (pH 7.6). The compartmentalised sections were then removed from the DAB-containing vessel and 20µl. of 15% H_2O_2 per 50ml. of the DAB solution was added and mixed. All presoaked sections were then immediately reacted in this solution (20-24°C.), with gentle agitation, for a further 30 minutes and then transferred through several rinsing solutions of 0.1M phosphate buffer (pH 7.4) before individually mounting on chrome alum - gelatin coated slides. If the sections were allowed to dry out at this stage then the tissue had a pitted appearance when examined under the microscope due to the presence of a large number of air bubbles. To overcome this the sections were mounted in a water-soluble mountant (Univert; Gurr) prior to drying out. This precluded the staining of these sections for Nissl substance. However, the tissue quality achieved by this procedure was very good. After drying, each section was methodically scanned microscopically for the presence of cell bodies containing HRP reaction product, using both bright and dark-field illumination. For each injected brain labelled cells were plotted by two independent observers and projected onto the appropriate plane of section taken from the atlas of König and Klippel.

Warning on the use of diaminobenzidine

Extreme care was taken when using this compound in view of its

alleged carcinogenic properties. The DAB was weighed out on a balance placed in a fume cupboard. Alternatively it was bought ready-weighed (100 mg.) During the weighing procedure a respirator and plastic gloves were always worn. Similar precautions were taken when working with the DAB solution. Following the tissue reaction procedures already described, Chloros solution was added to the remaining DAB solution to oxidise it into a less toxic compound, the structure of which is as yet undetermined.

RESULTS

The use of the HRP technique and a critical evaluation of the artifacts that can occur have been well described by Nauta et al., 1974. Their extensive description was used in the interpretation of the present results.

Distribution of HRP-labelled cells and artifacts following large injections of HRP into the corpus striatum

The injection sites in striatum were extensive and included parts of the overlying cortex. The injection site was characterised by a uniform dark brown stain when examined under the microscope. Towards the periphery of the injection site it was observed that numerous structures were present which contained fine granules of HRP. Nauta et al., 1974, reported that these were phagocytic cells. They appeared to be highly branched and were strand-like. It was noticeable that blood vessels, within 2-3 mm. of the injection site and ipsilateral to it, were stained a uniform dark brown colour. Red blood cells were also stained a dark brown colour. Cells labelled by retrograde transport of HRP were found in the zona compacta region of the SN and also in the parafascicular nucleus of the thalamus (Fig.39) as previously reported by Nauta et al., 1974; Kuypers et al., 1974. These cells were characterised by a fairly dense stippling with small reddish-brown granules of uniform size. In the zona compacta cell shown in Fig.40(A) it can be seen that this stippling was present mainly in the cell soma but it also extended into the dendritic processes of the cell. Apart from the presence of these fine granules

the retrogradely-labelled cells were free from other staining. However, in low magnification studies, under light-field illumination it was possible to confuse these retrogradely-labelled cells with endothelial cells which also contained HRP reaction product. As can be seen in Fig.40(B) these endothelial cells contained a slightly paler reaction product in vesicles of generally larger and more variable size than those observed in retrogradely-labelled cells. These two cell types could easily be distinguished at higher magnification.

In this initial experiment the material was examined under both light- and dark-field illumination and this procedure was continued throughout the course of the following experiments in the verification of suspected retrogradely-labelled cells. In the routine examination of sections it was found that the use of dark-field illumination, as recently reported by Kuypers et al., 1974, gave very clear-cut results when used at a magnification of x 150. The retrogradely-labelled cells in the parafascicular nucleus and zona compacta region of the SN are shown in Fig.41(A) and (B). Using this form of illumination it was easy to distinguish labelled endothelial cells from HRP-labelled neurones since the former were characterised by a reddish granular appearance. Thus, it was found to be easier to examine all the sections initially under dark-field illumination and compare the labelled neurones distribution with that found under light-field illumination. It was found that there was very good agreement between the two methods.

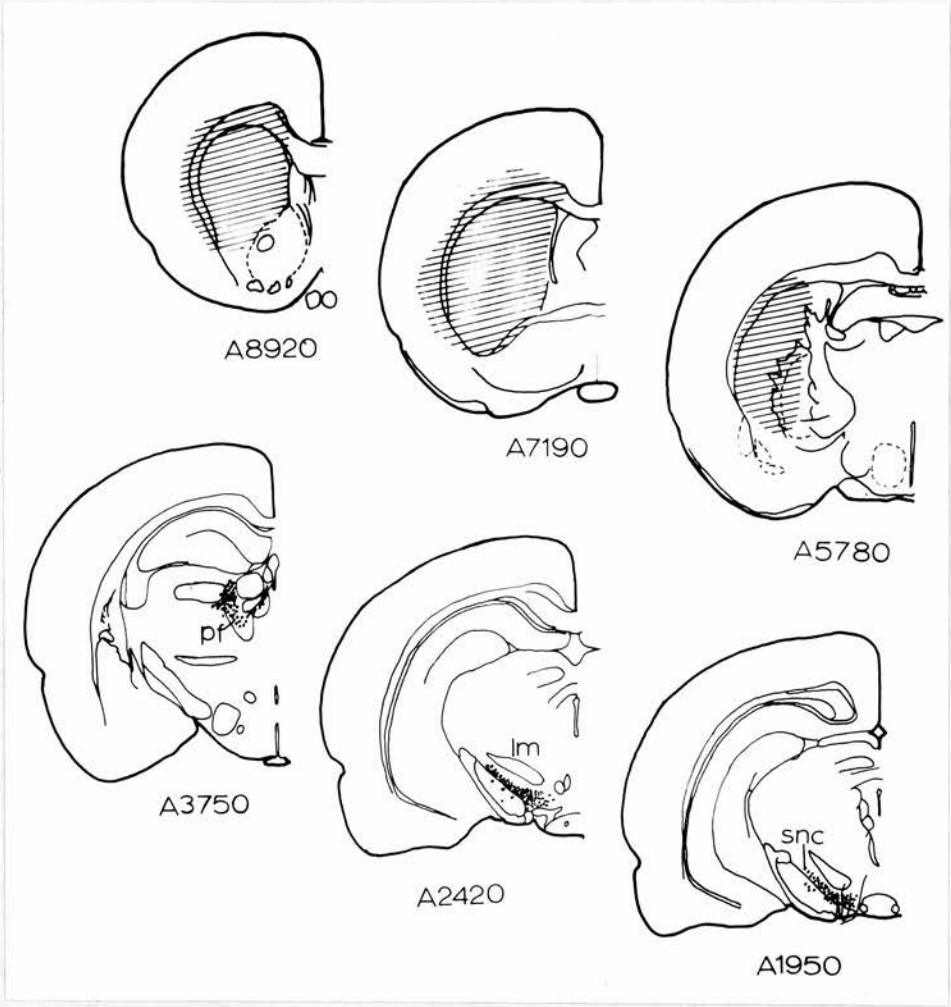

Figure 39

Distribution of neuronal cell bodies containing horseradish peroxidase (HRP) reaction product in an adult rat after HRP injection in the region of the corpus striatum (1 day survival). The hatching in the upper three sections illustrated above marks the extent of the injection site. Each dot marks the position of one cell. All sections were taken from the atlas of König and Klippel.

Abbreviations: lm, lemniscus medialis; pf, parafasicular nucleus; snc, substantia nigra, zona compacta.

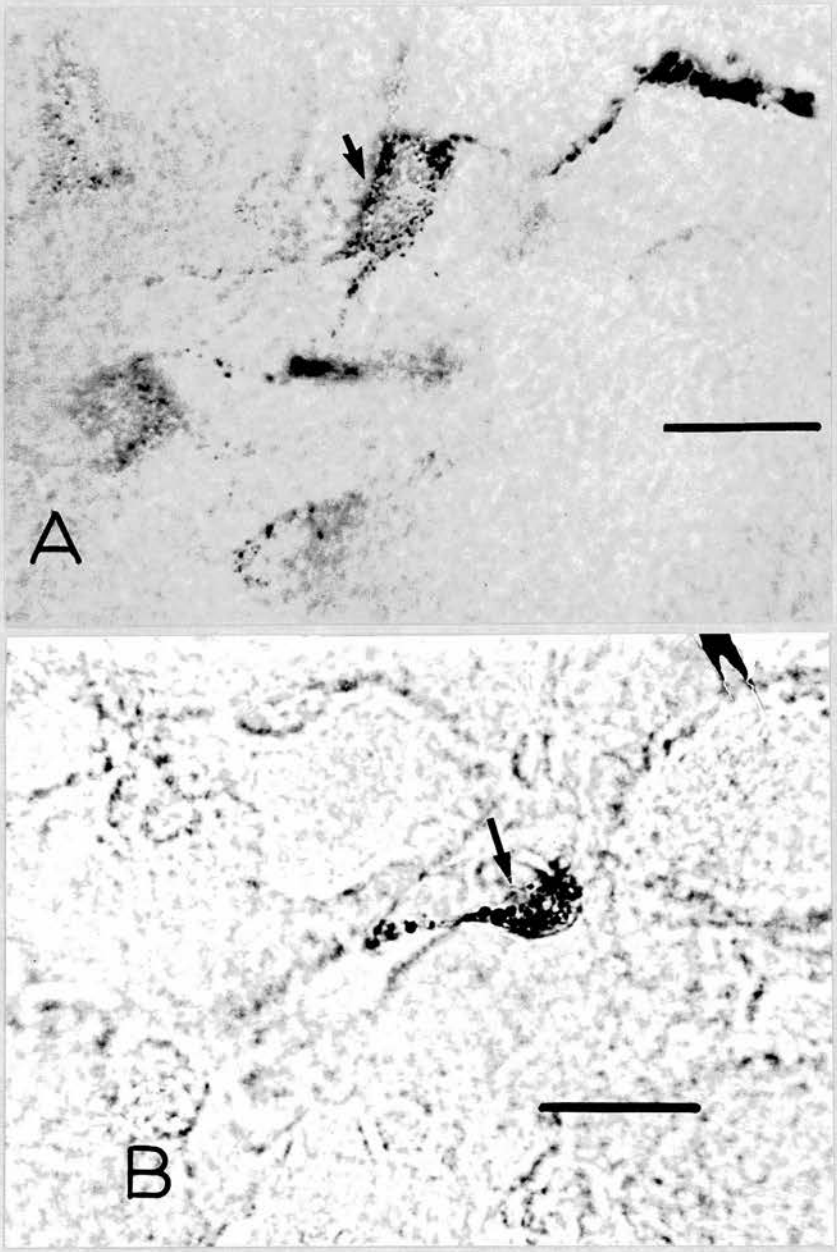

Figure 40

A. A neurone in the substantia nigra, zona compacta (arrow), thought to have been labelled by uptake and retrograde transport of HRP injected in the caudatoputamen. The HRP is confined to granules of equal size, localised principally in the peripheral region of the cell and in the primary dendritic processes.

B. Endothelial cell containing HRP reaction product (arrow). Note that the product is localised in vesicles of variable size.

Scale bars are 20μm.

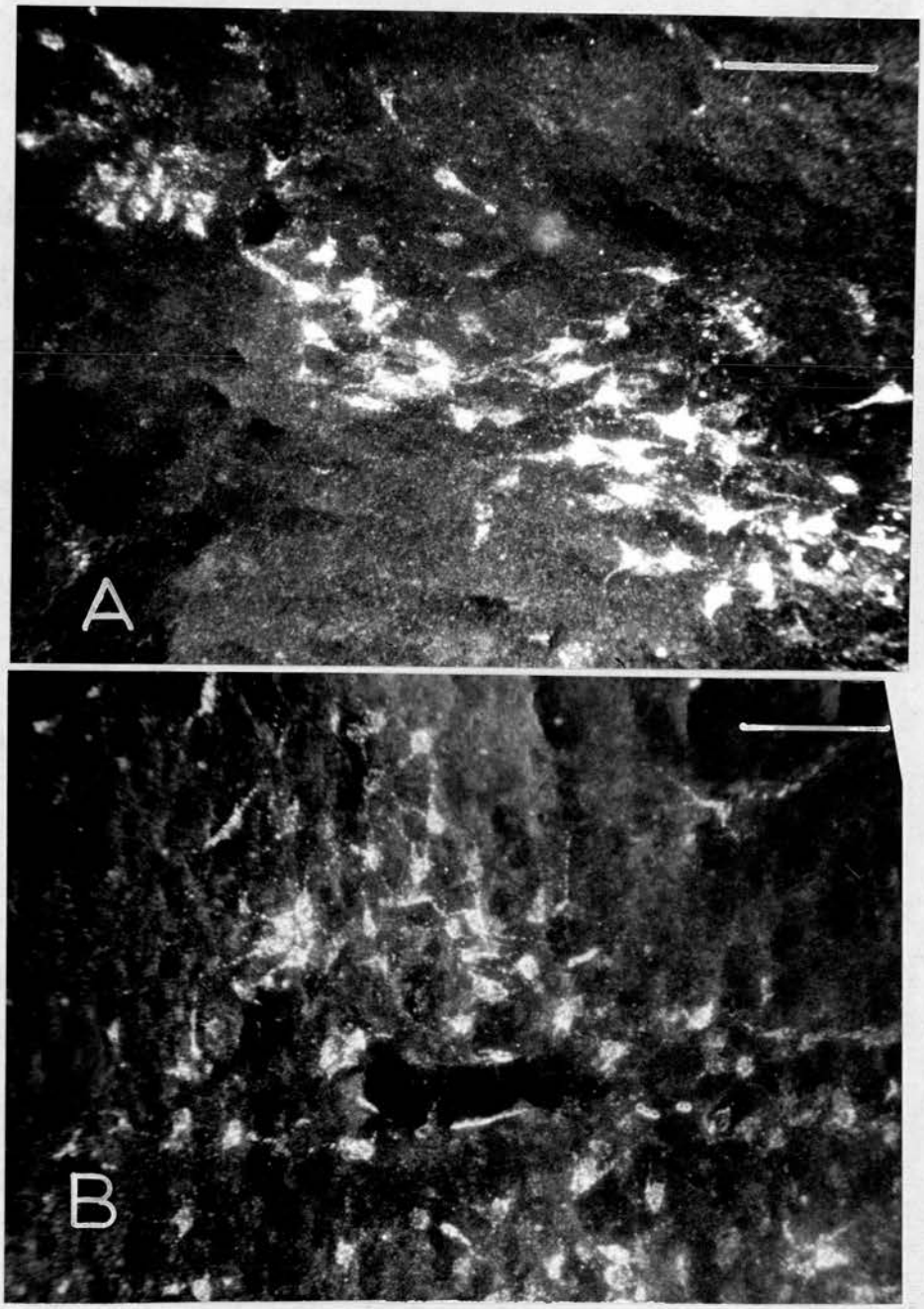

Figure 41

Photomicrographs (dark-field illumination) showing neuronal cell bodies containing HRP reaction product.

A. HRP-positive neurone in the left SN after HRP injection into the left corpus striatum (1 day survival).

B. Presence of HRP-positive neurones in the left parafascicular nucleus in the same animal.

Scale bars are 100μm.

Distribution of retrogradely labelled neurones following HRP injection into the region of the zona reticulata region of the SN.

Two animals with injection sites well localised to the zona reticulata region are included in this section. Although both brains had a similar distribution of labelled cells there were some differences, especially with regard to labelling of cells in the cerebral cortex. Fig.42 is a schematic diagram showing the injection site and distribution of labelled cells in one animal (ZRI). The other successfully injected animal (ZR2) will be described with reference to ZRI.

The most intense staining in ZRI was found in the zona reticulata region and this structure was quite well stained throughout its rostral-caudal extent. The staining did extend dorsally, almost to the medial lemniscus but it was much less intense than that present in the zona reticulata. Included in this zone of weaker staining was the zona compacta region. It was observed that some cells in the zona compacta region appeared to be labelled in an identical manner to that found in retrogradely-labelled cells described above (Fig.43B). These are not included in the summary diagram due to the difficulty in establishing whether they were in fact retrogradely-labelled or not. The interpretation of the results was otherwise fairly clear-cut. Identified retrogradely-labelled neurones were found in the region of the dorsal raphe nucleus, corpus striatum, globus pallidus and the lateral frontal cortex. Labelled cells were also observed in the subthalamic nucleus (Fig.43A). Individual photomicrographs of HRP-positive neurones observed in the other areas, excluding the dorsal raphe nucleus, are shown in Figs.44A-C.

Peroxidase activity was also present bilaterally in the region of the arcuate nucleus (Fig.44D). A similar finding has recently been reported by Sherlock, Field and Raisman, 1975, and is thought to be due to endogenous peroxidase activity. This activity was present in all animals studied in this present investigation.

The distribution of retrogradely-labelled cells in case ZRI was similar to that observed in the other zona reticulata injected animal, (ZR2) apart from a difference in the extent of cortical cell labelling. ZR2 had a more intense labelling of the frontal cortex and a few labelled cells, also ipsilateral to the injection site, were found in a narrow band in the lateral cerebral cortex as far caudal as the site of injection. It is unlikely that the presence of labelled cortical cells was due to the spread of injected HRP into the overlying cortex since no staining was observed in that region. Thus, it is possible that the difference in labelling may represent a slight difference in the site of injection. In both animals it was noticeable that the subthalamic nucleus was stained light brown and granules of HRP were present in this region. This staining was continuous from the zona reticulata to the level of the subthalamic nucleus and occupied the medio-ventral surface of the crus cerebri. Some staining of the crus cerebri was also observed. The presence of staining and HRP grains in the subthalamic nucleus did present a problem in establishing whether or not retrogradely-labelled cells were localised in this area. However, following examination at high power magnification, under light field illumination, both observers agreed that labelled cells were present in this nucleus (Fig.43A).

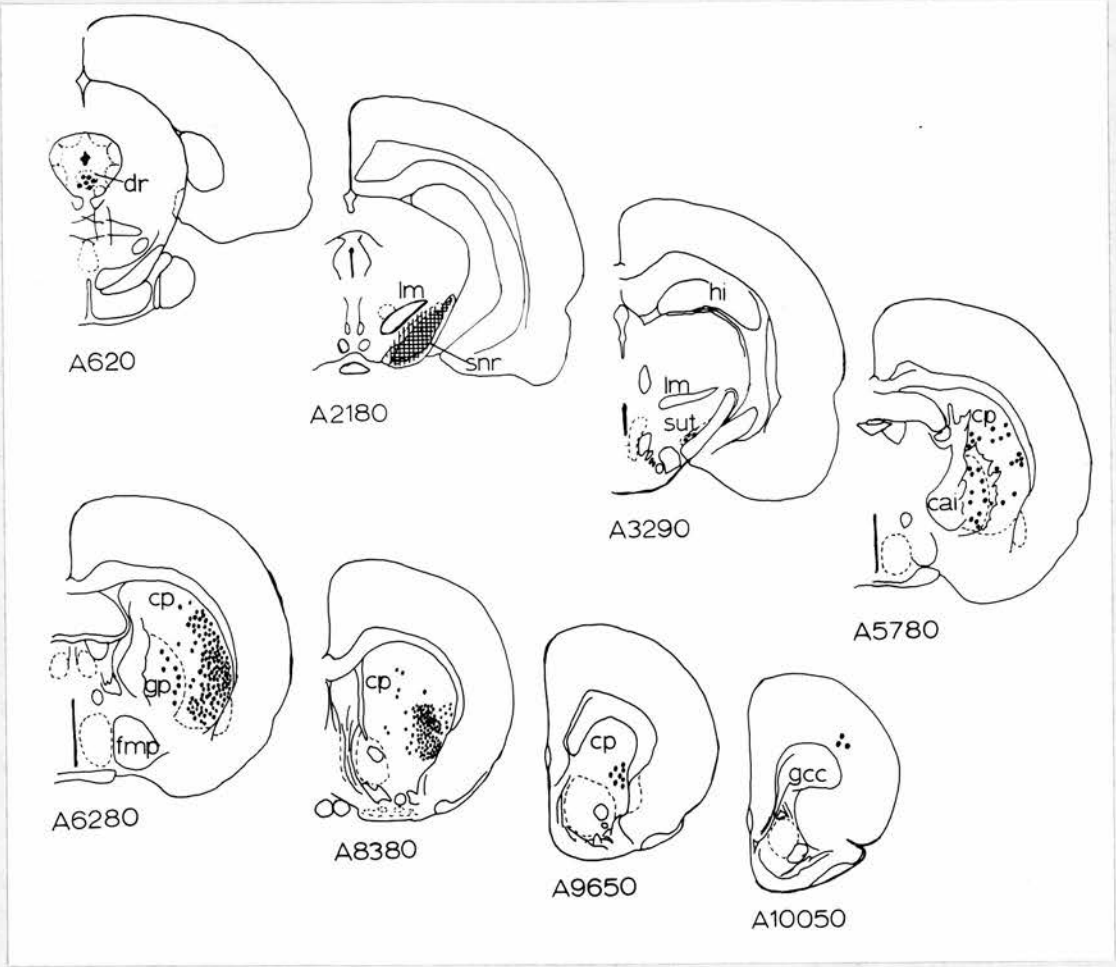

Figure 42

Diagrammatic representation of the injection site and distribution of retrogradely-labelled cells in case ZR1. The varying intensity of brownish reaction product is indicated by hatchings in section A2180. Each dot indicates one labelled cell. All sections were taken from the atlas of König and Klippel (indicated by number underneath).

Abbreviations: cai, internal capsule; cp, caudatoputamen; dr, dorsal raphe; fmp, fasiculus medialis prosencephali; gcc, genu corporis callosi; hi, hippocampus; lm, lemniscus medialis; snr, substantia nigra zona reticulata; sut, subthalamic nucleus.

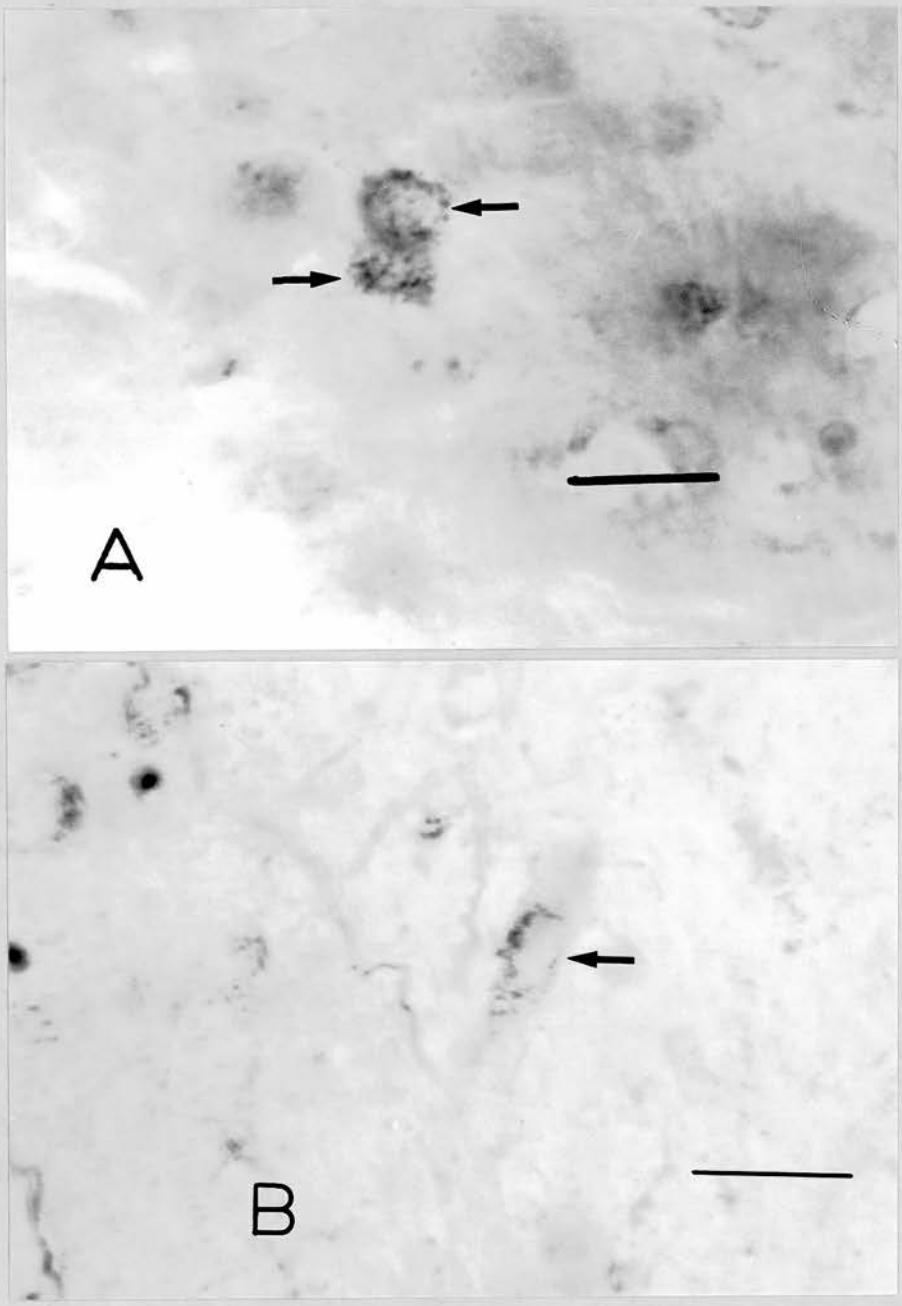

Figure 43

Photomicrographs (bright-field illumination) of cells thought to have been labelled with HRP reaction product.

A. Labelled cells (arrows) in the left (injected side) subthalamic nucleus in case ZR1. These cells were indistinguishable from neurones labelled by the retrograde transport of HRP and are included in the summary diagram shown in Fig.42.

B. Labelled cell (arrow) in the left zona compacta region in case ZR1. The granular appearance of these cells was indistinguishable from that observed in retrogradely-labelled cells. These cells are omitted from Fig.42. Scale bars = 20μm.

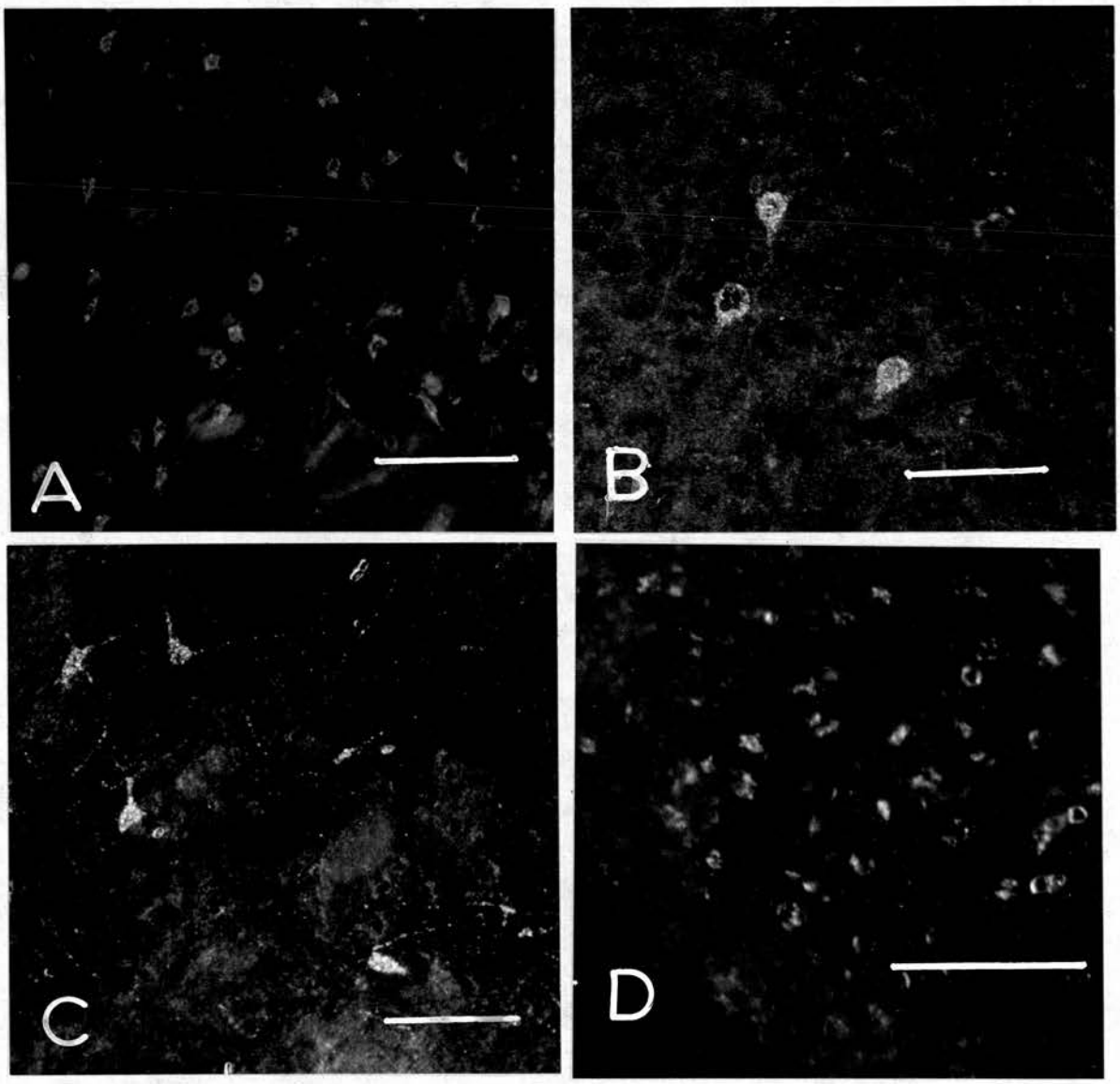

Figure 44

Photomicrographs (dark-field illumination) showing HRP reaction product in cell bodies.

A. HRP-positive neurones in the rostral region of the left striatum after HRP injection in the left zona reticulata (case ZR1)

B. Labelled neurones in the left frontal cortex in the same animal.

C. HRP-positive neurones in the left globus pallidus of case ZR1.

D. Endogenous HRP activity found bilaterally in cells in the region of the arcuate nucleus. Note the rough granular appearance of the reaction product. Scale bars represent 100μm.

Distribution of retrogradely-labelled neurones following HRP injections well localised to the zona compacta region of the SN

The angular injection approach and the careful retraction of the needle during the course of an injection resulted in HRP being well localised to the zona compacta region in 4 rats. In all these animals the needle tract was along the zona compacta and this area was very intensely stained with HRP. Fig.45 is a photomicrograph illustrating the injection needle tract and maximum extent of HRP staining in a zona compacta injected animal (ZC3). Fig.46 shows schematically this injection site in the zona compacta and the distribution of retrogradely-labelled cells found in this animal (ZC3). The zona compacta region was very intensely stained in this animal with only a minimal spread of HRP to the zona reticulata region. In the rostral and caudal aspects of the zona compacta, where HRP staining was less intense, it was consistently observed that labelled cells were present identical to those described previously as being retrogradely-labelled. In view of the high concentration of HRP surrounding these cells it proved very difficult to establish whether these cells were retrogradely-labelled or not. Due to this difficulty these "labelled" cells were omitted from Fig.46. In contrast, no such labelled cells were present in the zona reticulata region.

Rostral to the injection site it was observed that a small "spur" of light uniform HRP staining extended to the level of the subthalamic nucleus. This staining was present on the medio-ventral surface of the crus cerebri and extended medially for about 0.5mm. into the H_2 Field of Forel.

The distribution of retrogradely-labelled cells found in ZC3 (Fig.46) was similar to that found in the other three animals injected in the zona compacta (ZC2, ZC4, ZC14) but there were noticeable differences between animals in the number of labelled cells found in a given area. The distribution of labelled-cells in rat ZC3 best illustrates the pattern of labelling following a zona compacta HRP injection and includes all labelled areas found in the other similarly injected animals. Positively identified retrogradely-labelled cells were found in the region of the dorsal raphe nucleus, superior cerebellar peduncle, lateral hypothalamus, the magnocellular region of the paraventricular nucleus, tail of the striatum, globus pallidus and the interstitial nucleus of the stria terminalis. Individual photomicrographs of HRP-positive neurones from these areas are shown in Figs.47A and B; 48A,B and C; 49A and B.

In all animals studied it was observed that evenly stained fibres (Fig.50) apparently arising from the substantia nigra, coursed rostrally within the medial forebrain bundle and entered the medial tip of the crus cerebri before fanning out into the striatum. No labelled cells or fibres were found contralateral to the injection site.

In a bid to summarise the findings from the zona compacta injection sites and compare these with those obtained following zona reticulata injections it was decided to select planes of section in which the greatest number of labelled cells were found and which best illustrated the labelled cell distribution following a particular

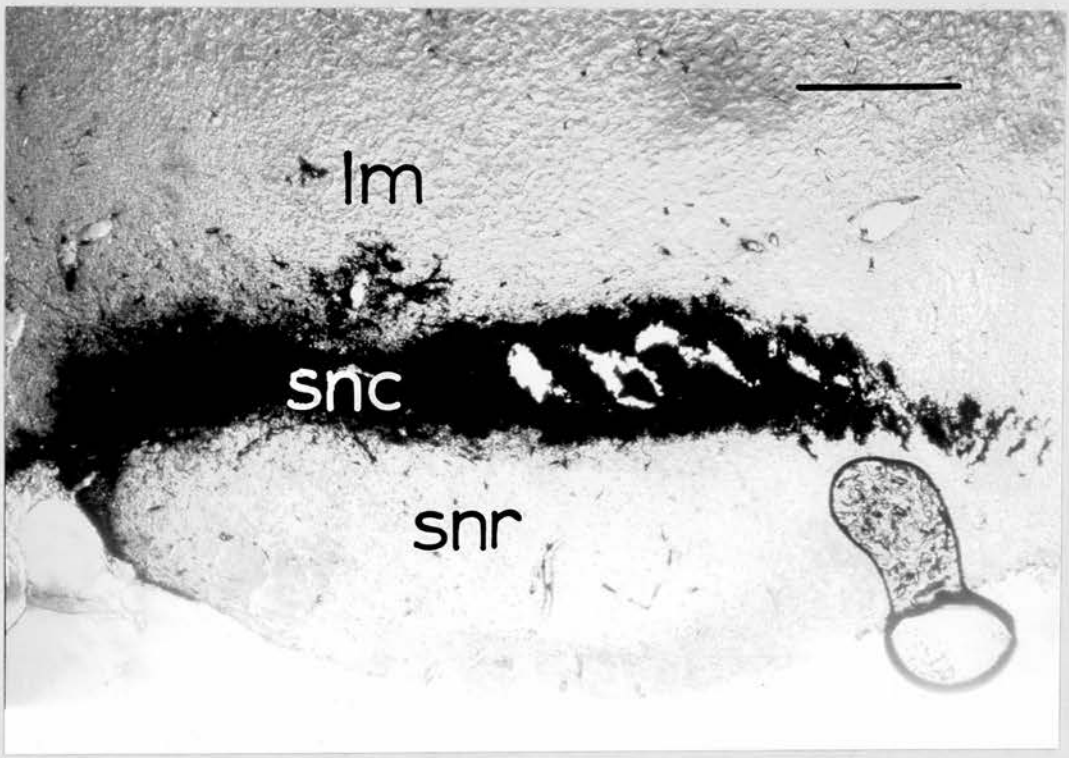

Figure 45

Low power photomicrograph showing HRP injection site in the zona compacta region of the SN (case ZC3). The HRP was found to be well-localised to the zona compacta, following slow retraction of the needle during the injection procedure. An air bubble is present in the lower right of the micrograph.

Scale bar = 0.5mm.

Abbreviations: lm, lemniscus medialis; snc, substantia nigra, zona compacta region; snr, substantia nigra, zona reticulata region

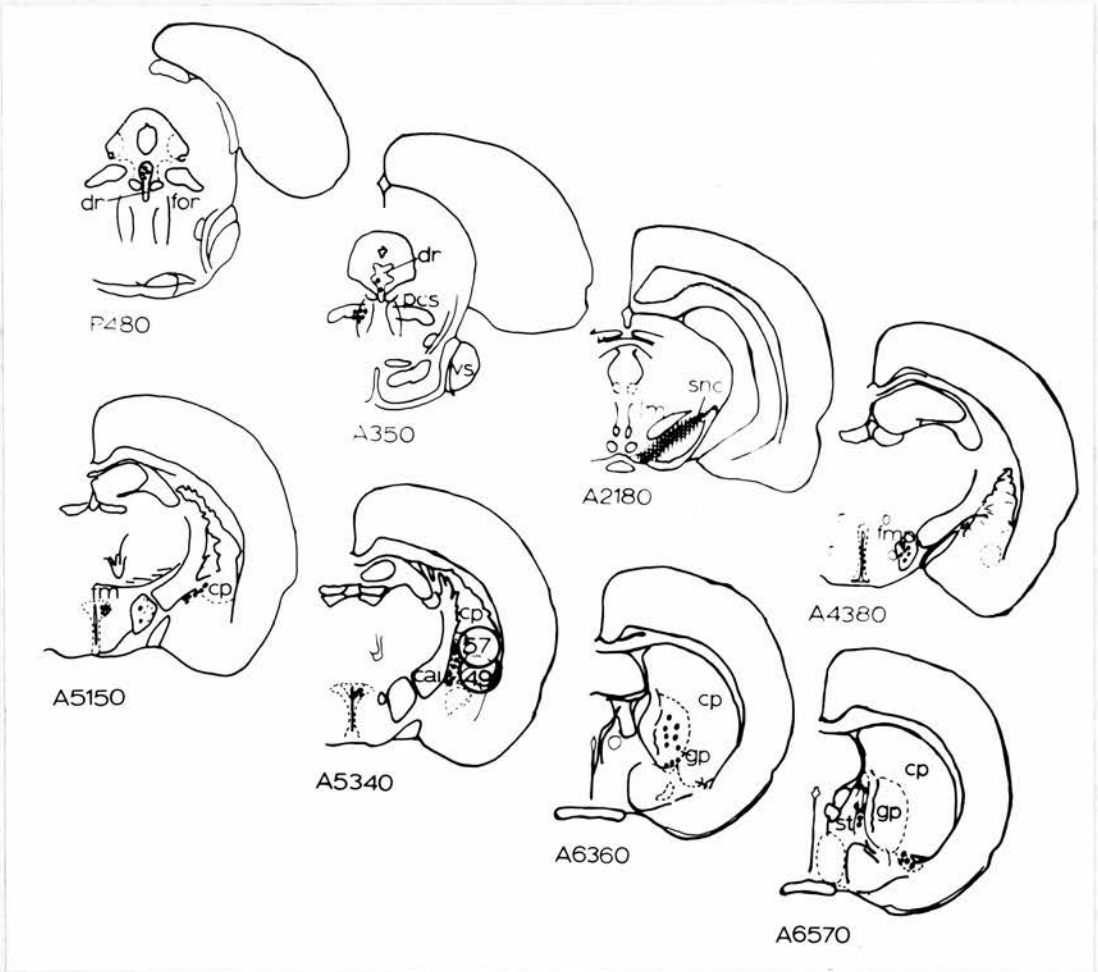

Figure 46

Diagrammatic representation of the injection site and distribution of retrogradely-labelled cells in case ZC3. The varying intensity of brownish reaction product, observed at the injection site, is indicated by hatchings. Each black dot indicates the position of a labelled cell. In the tail of the striatum shown in section A5340 labelled cells were counted in 2 fields of view (indicated by circles). The number in each circle refers to the number of labelled cells counted. All sections were taken from the atlas of König and Klippel.

Abbreviations: cai, internal capsule; cp, caudatoputamen; dr, dorsal raphe; fm, nucleus paraventricularis, pars magnocellularis; fmp, fasiculus medialis prosencephali; for, reticular formation; gp, globus pallidus; lm, lemniscus medialis; pcs, pedunculus cerebellaris superior; st, stria terminalis; snc, substantia nigra, zona compacta.

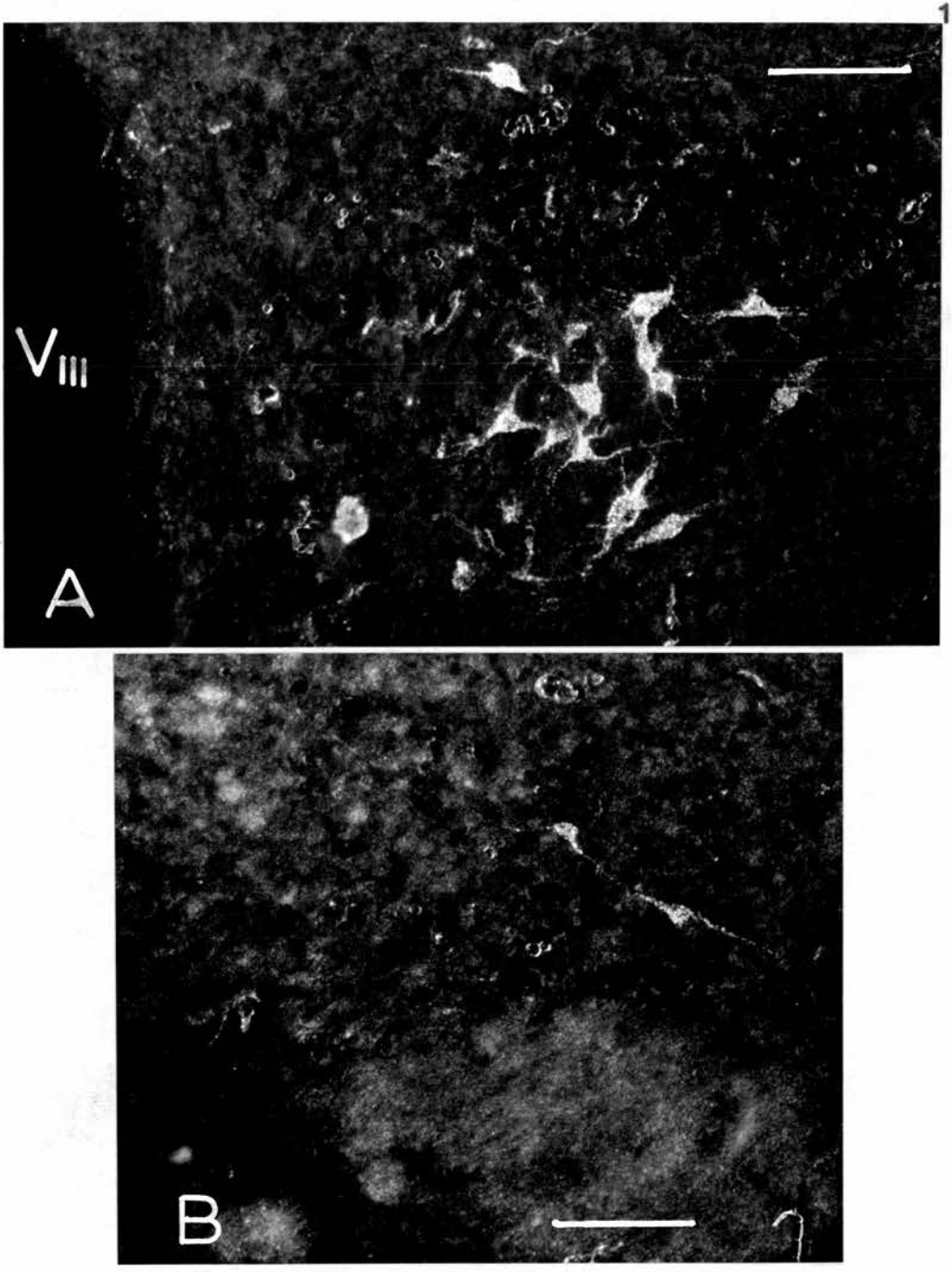

Figure 47

Photomicrograph (dark-field illumination) showing HRP reaction product in neuronal cell bodies.

A. HRP-positive neurones in the region of the left paraventricular nucleus, pars magnocellularis, after HRP injection into the left zona compacta area (case ZC3) V III, third ventricle.

B. HRP-labelled neurones in the left lateral hypothalamic region in the same animal. Scale bars are 100μm.

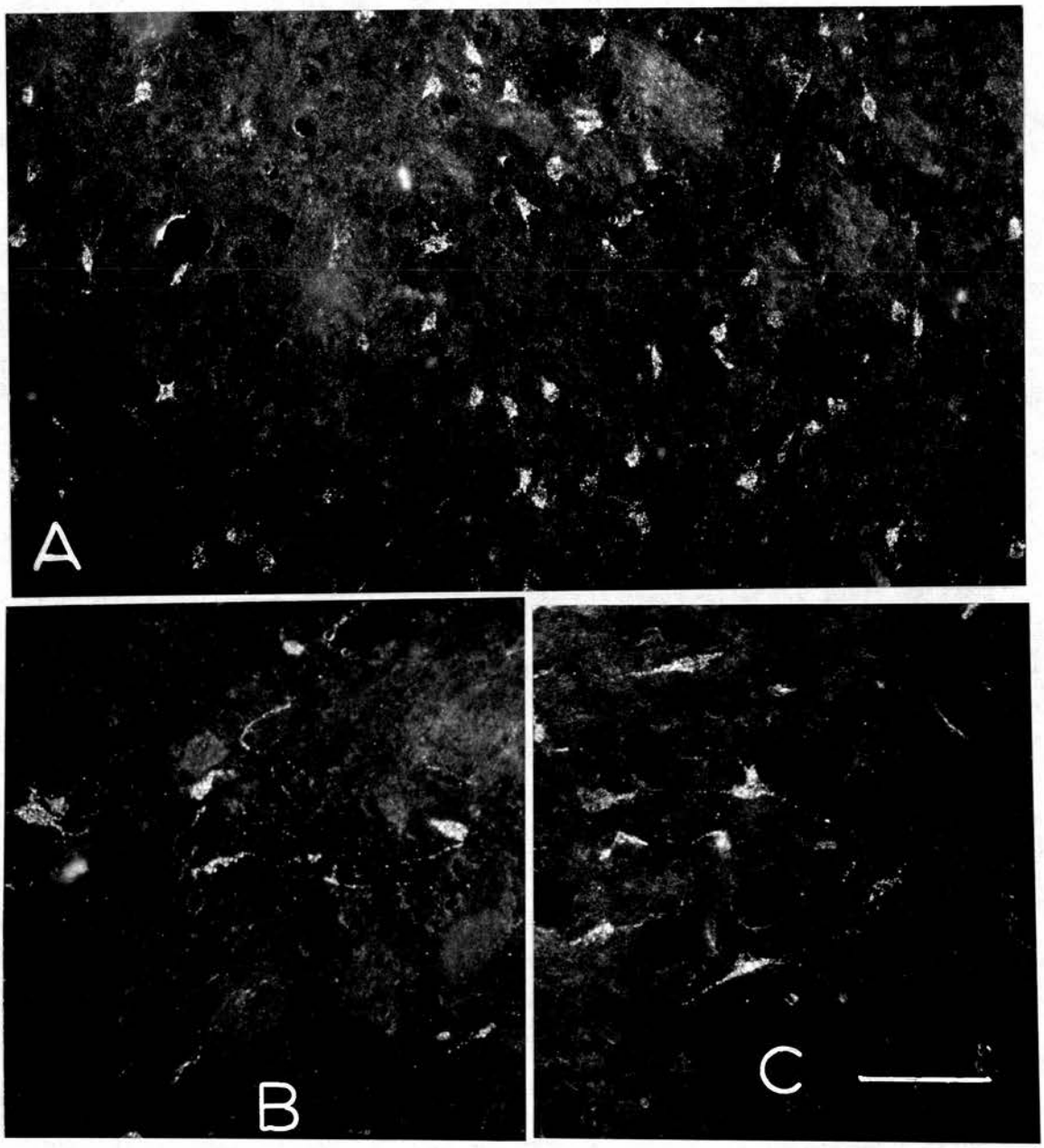

Figure 48

Photomicrographs (dark-field illumination) showing HRP reaction product in neuronal cell bodies.

A. HRP-positive neurones in the caudal region of the left corpus striatum in case ZC3.

B. HRP-labelled neurones in the caudal region of the left globus pallidus in case ZC3.

C. HRP-positive neurones in the left interstitial nucleus of the stria terminalis in the same animal.

The scale bar = 100μm. and applies to all 3 parts of this figure.

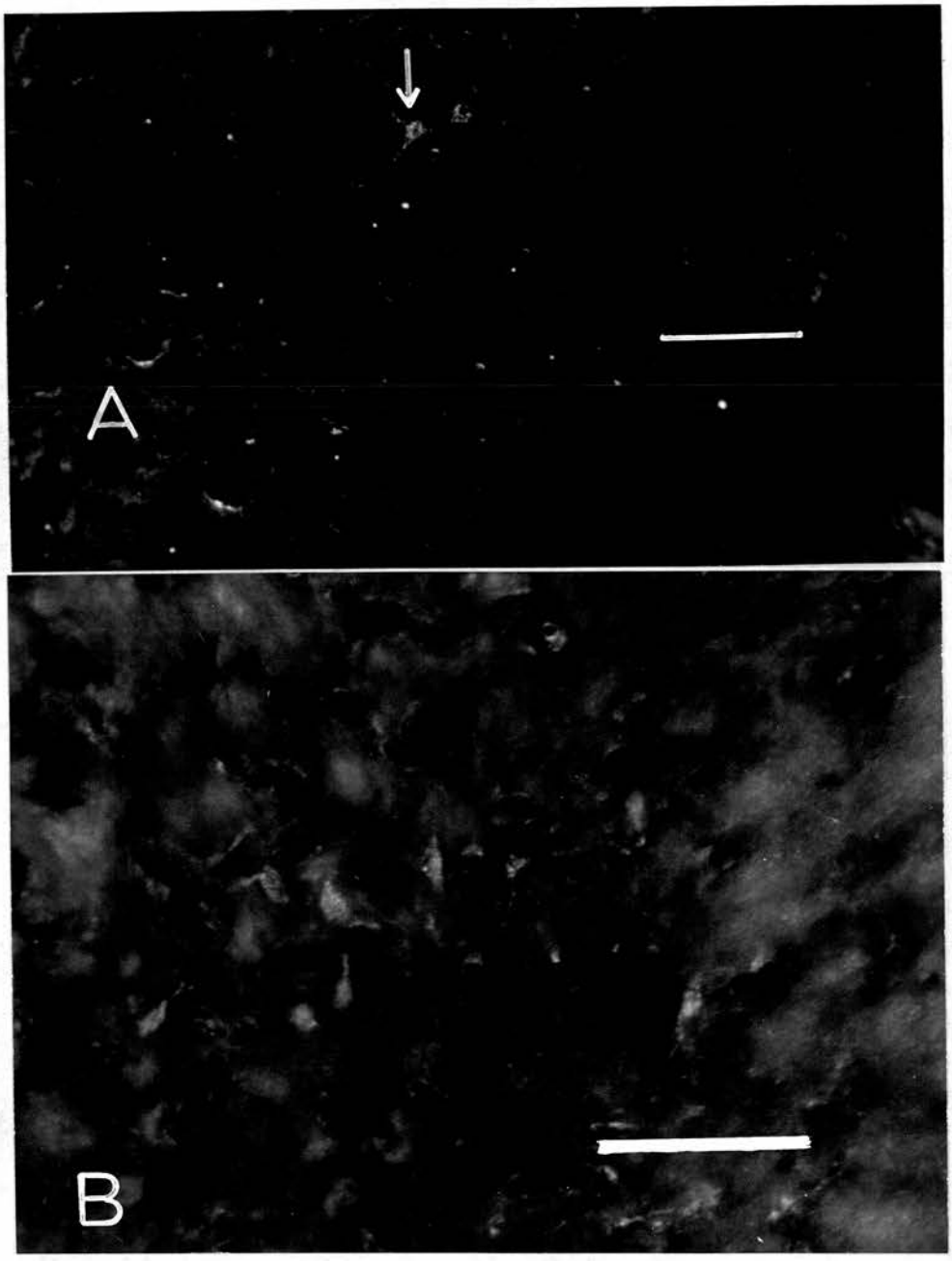

Figure 49

Photomicrographs (dark-field illumination) showing HRP reaction product localised to neuronal cell bodies.

A. 2 HRP-positive neurones (1 indicated by the arrow) in the region of the dorsal raphe nucleus in case ZC3.

B. A cluster of HRP-positive neurones in the region of the superior cerebellar peduncle. The surrounding myelin (light gray, diffuse areas) partly mask the presence of the labelled neurones.

Scale bars = 100μm.

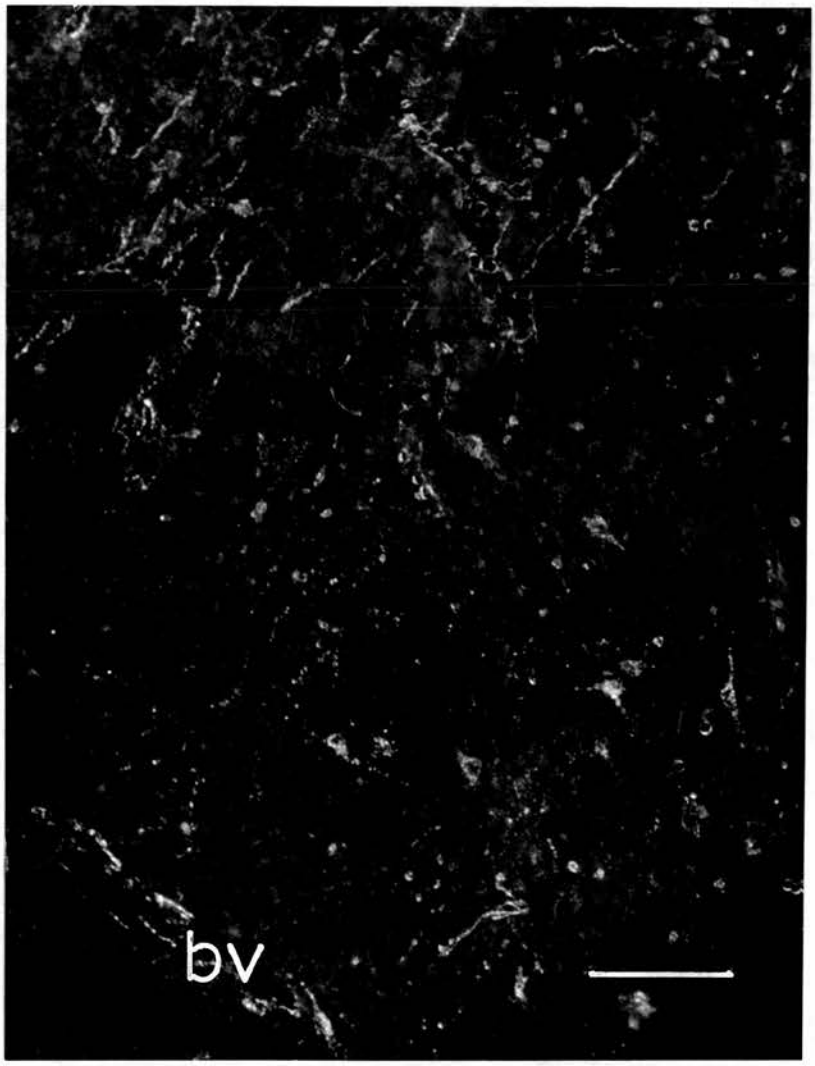

Figure 50

Photomicrograph (dark-field illumination) illustrating the presence of evenly stained fibres and HRP-positive neurones in the lateral hypothalamic region. In the dorsal region of the lateral hypothalamus (upper part of photomicrograph) the stained fibres appear as thin, light-coloured strands. Characteristic HRP-positive neurones can be seen in more ventral regions of the photomicrograph. A blood vessel (bv) can be seen surrounded by HRP-labelled endothelial cells. Scale bar = 100μm.

injection. All labelled cells were counted in these sections in each brain and the results are summarised in Table 1. Although there are slight differences between animals it is possible to comment in general about the differences of labelling between the two groups of injected animals. Following zona compacta injection labelled cells were predominantly found in the tail of the striatum with virtually none present in the head of this nucleus. This is in contrast to the labelling in striatum following zona reticulata injection where the rostral parts of this nucleus are very heavily labelled. Zona compacta injection also resulted in labelling of cells in the lateral hypothalamus and in the region of the para-ventricular nucleus. Rostrally, labelled cells were found in the interstitial nucleus of the stria terminalis and a few were present in the extreme ventral region of the striatum. These areas were not labelled following injection into the zona reticulata. Another notable difference between the two injection sites was the presence of labelled cells in the lateral frontal cortex following zona reticulata injections. The presence of the other cortical cells labelled in ZR2 mentioned previously, are not indicated in Table 1. No cortical labelling was observed following zona compacta injections. In three zona compacta injected animals from which material was available a small cluster of labelled cells was found in the medial region of the superior cerebellar peduncle. Only one zona reticulata injected animal was studied in this caudal region and labelled cells were absent although they were present in the dorsal raphe nucleus.

TABLE 1.

Plane of Section	Brain Area	Rat number ZC3	ZC4	ZC2	ZC14	ZR1	ZR2
P480	Dorsal Raphe	5	2	4	1	0	-
A350	Dorsal Raphe	2	5	1	-	3	-
	PCS	6	4	6	-	0	-
A3290	Subthalamic Nucleus	0	0	0	0	3	3
A4380	Lateral hypothalamus	7	3	10	8	0	0
A5150	Lateral hypothalamus	2	0	0	0	0	0
	Paraventricular nucleus	10	6	3	2	0	0
	Striatum	6	17	4	16	0	0
A5340	Paraventricular nucleus	2	13	0	6	0	0
	Globus pallidus	8	2	14	1	0	30
	Striatum	116	25	106	20	0	115
A5780	Globus pallidus	4	2	8	3	12	12
	Striatum	0	0	11	2	16	154
A6280	Globus pallidus	10	2	2	0	8	7
	Striatum	1	2	0	0	174	368
A6790	Stria terminalis	8	1	9	2	0	0
	Striatum	4	4	2	0	170	342
A8380	Striatum	0	0	0	0	114	210
A9650	Striatum	0	0	0	0	8	0
A10050	Frontal cortex	0	0	0	0	3	5

HRP-positive neurone distribution in rat brain following HRP micro-injections into the substantia nigra. Animals ZC3-ZC14 were injected in the zona compacta region whereas ZR1 and ZR2 were injected in the zona reticulata. Numbers refer to labelled neurones observed in the various brain areas, ipsilateral to the injection site. All planes of section were taken from the atlas of König and Klippel.

Abbreviation: PCS, pedunculus cerebellaris superior

DISCUSSION

The HRP technique proved to be quite straightforward in its application but considerable care was required in the interpretation of the results. Artifacts, including labelled endothelial and other phagocytic cells were always present as previously described in detail by Nauta et al., 1974, but in most instances these could be easily distinguished from the characteristic stippled appearance of the retrogradely-labelled cells. The initial study on the afferent pathways to the corpus striatum served to acquaint the author with the various interpretative difficulties involved and also to compare the resulting labelled-cell distribution with that reported by Nauta et al., 1974; Kuypers et al., 1974 and Ljungdahl et al., 1975. In particular, the distribution and appearance of labelled cells in the zona compacta region of the SN was in good agreement with the findings of these workers, thus establishing that the adopted HRP histochemical protocol was capable of demonstrating the presence of retrogradely-transported enzyme in cell bodies quite far distant from the site of injection. Immuno-histochemical studies, utilising a fluorescent-tagged tyrosine hydroxylase antibody in combination with intra-striatal injections of HRP have shown that the HRP-containing cells in the SN also contain TOH (Ljungdahl et al., 1975). This suggests that these cells are dopaminergic in nature and that they are retrogradely-labelled by HRP. Although no labelling was detected in the zona reticulata in this initial study at the light microscope level it has been reported from an electron microscope (EM) study (Nauta, Kaiserman-Abramof and Lasek, 1975) that labelled terminals

are present in this region following intra-striatal HRP injection. This gives rise to the interesting possibility that the technique could be used at the EM level to trace both anterograde and retrograde transport of HRP from the same injection site.

The presence of endogenous peroxidase was confined largely to erythrocytes remaining in the perfused brain tissue and to small cells in the arcuate nucleus. The rough granular appearance of the latter reaction product, also reported by Sherlock, Field and Raisman, 1975, was easily distinguishable from that found in retrogradely-labelled neurones. These workers reported that this activity found in the arcuate nucleus was restricted to astrocytes. Although no non-injected animals are included in this present study, the ipsilateral location of retrogradely-labelled cells and the conspicuous absence of similarly labelled cells in the contralateral side, does strongly suggest that all the identified cells were labelled by the retrograde transport of HRP and not by endogenous peroxidase activity. Recently, however, it has been reported that endogenous activity is present in neurones in some regions of the extrapyramidal system (globus pallidus, SN and red nucleus) of the squirrel monkey (Wong-Riley, 1975, 1976). This activity was confined largely to iron-rich nucleur groups and the stippled appearance of the labelled neurones, as visualised at the light microscope level, was indistinguishable from retrogradely labelled neurones. Although this form of activity does not appear to present any problems in the rat it is obviously important in some species to compare the results from injected animals with those from non-injected controls. Before discussing further the

the findings in this present study it is necessary to include some of the more serious shortcomings of the method.

Although the majority of workers agree on the histological characteristics of retrogradely-labelled cells it is quite apparent that there is uncertainty as to whether this labelling results solely from uptake by axon terminals. Nauta et al., 1974, suggested that uptake of HRP by traumatised axons and cells resulted in a characteristic uniform staining of cell somata. This is at variance with other studies from the peripheral nervous system. Uptake and retrograde transport of HRP have been demonstrated in the cut vagus nerve (De Vito, Clausing and Smith, 1974). The resulting stippled appearance of the cell bodies within the central nuclei of these nerves suggested that this form of labelling was indistinguishable from that reported for undamaged neurones. Within the CNS it is difficult to transect a given neuronal system and demonstrate HRP uptake and labelling damaged neurones without clouding the results by the simultaneous uptake and transport of HRP in undamaged neurones. However, the time is ripe for studies of this nature on well-defined neuronal systems. It should be possible to transect a compact bundle of axons and introduce, ideally by iontophoresis, a small amount of HRP into the region of the cut axons. The resulting labelled cell distribution could be compared with that observed following similar injections of enzyme into the intact bundle. Although it is likely that damaged axons in the CNS do retrogradely transport HRP there is now evidence that undamaged axons, passing through an injection site, do not accumulate HRP to any significant extent. This has been shown in unmyelinated fibres in the chick stratum opticum following tectal

injections (La Vail and La Vail, 1975) and myelinated fibres coursing through the corpus striatum (Nauta et al., 1974). With the present limited extent of knowledge it is uncertain whether this applies to all axon types in the CNS.

In the past 4 years numerous established central pathways have been convincingly demonstrated by the HRP technique. Thus, it would appear that most neuronal types in the CNS are capable of retrogradely transporting HRP. The most notable exception to this is the lack of neocortical cell labelling following intra-striatal HRP injections (Nauta et al., 1974). In this present study no convincing labelled cells were detected in the cortex following very similar striatal injections. In view of this finding it is obvious that negative results with this technique must be interpretated with the utmost caution.

The present findings following zona reticulata microinjections of HRP were in agreement with those reported in the cat (Grofova, 1975) following similar injections. Many labelled cells were found in the corpus striatum throughout its rostral-caudal extent with relatively fewer labelled cells present in the globus pallidus. Further studies using the electron microscope would further help to characterise these different cell types. Grofova's study was confined to the mesencephalon, thalamus and basal ganglia but this present, more extensive study also showed that labelled cells could be identified in the forebrain and pontine regions, namely in the frontal cortex and dorsal raphe nucleus. Recently it has been shown that SN neurones, predominantly located in

the zona reticulata region, are inhibited by electrical stimulation in the region of the medial raphe nucleus and also by iontophoretic application of 5-HT, (Dray, Gonye, Oakley and Tanner, 1976). It is possible, as these authors themselves suggest, that these electrical stimulation effects could have been mediated by stimulation of dorsal raphe axons passing through or alongside the medial raphe nucleus. It would certainly be interesting to investigate whether nigral neurones were influenced by dorsal raphe stimulation. Although no labelled cells were present in the medial raphe nucleus of the animal studied it is obvious that additional zona reticulata injected animals are required. This would also help to clarify the discrepancy between animals with regard to the extent of cortical labelling. However, from the two animals studied it does appear that a discrete projection exists from the lateral frontal cortex to the zona reticulata region. The existence of a pathway from the pre-frontal sulcal cortex to the SN has recently been reported (Clavier and Corcoran, 1976).

The retrogradely-labelled cell distribution following well-localised injections to the zona compacta region was strikingly different from that observed with zona reticulata injection sites. This strongly supports the suggestion that the distribution of cells labelled with HRP correlates most closely with the immediate centre of the injection site (Jones and Leavitt, 1974; Jones, 1975; La Vail, 1975). Labelled cells in the striatum were predominantly present in the more caudal aspect (tail) of this nucleus. This part of the caudate has been the subject of 1 previous detailed study (Szabo, 1972)

in the monkey. It was reported that the efferents from the tail of the caudate projected to the ventro-lateral region of the zona reticulata. However, in the rat it does appear that a projection to the zona compacta region may exist. A moderate number of labelled cells were present in the globus pallidus, particularly in the caudal region. These findings suggest that these brain areas do project to the zona compacta region although they do not rule out the possibility that uptake by terminals in the dorsal zona reticulata may have contributed to the overall labelled cell distribution.

The presence of labelled cells in the lateral hypothalamus supports the existence of a projection from this region to the zona compacta region. It is clear from the autoradiographic results presented in Chapter III that labelled fibres, arising from cell bodies in the lateral hypothalamus, course through the zona compacta. It is likely that a few of these axons would be damaged to a certain extent by the pressure injections employed here and, HRP uptake by severed axons may have contributed to the labelling pattern within the lateral hypothalamus. This possibility accentuates the difficulty in obtaining conclusive evidence about short neural projections in the CNS utilising the present tracing techniques at the light microscope level. Probably the best method available is EM autoradiography, where it can be shown whether labelling is in terminals or in axons of passage (due to the presence of radioactive leucine).

The presence of large labelled cells in the region of the paraventricular nucleus was a surprising finding. The present study

provides only sparse histological details of these labelled neurones and it is apparent that a further study of these neurones is required. It is known that neurones in this area contain oxytocin and vasopressin (Bisset, Errington and Richards, 1973; Zimmerman, 1974; Swaab, Pool and Nijvelot, 1975) and that they can be antidromically invaded by electrical stimulation in the region of the pituitary stalk (Freund-Mercier, Richard and Miro, 1975). These findings indicate that these neurones are responsible for the release of either vasopressin or oxytocin in the median eminence. Three types of cell have been characterised electrophysiologically in the paraventricular nucleus (Freund-Mercier et al., 1975) and of these, two types were antidromically invaded by pituitary stimulation. It is possible that the neurones labelled in the present study were of the uninvaded category. However, central projections of peptide-containing neurones to areas outwith the hypothalamus should not be discounted (Renaud, 1976). It would be tempting in the future to combine immunohistochemical studies on the neurones of the paraventricular nucleus with the HRP technique in a bid to establish whether the HRP labelled cells reported in this study were neurosecretory in nature.

One disquieting feature of this present finding is the remote possibility that the paraventricular neurones may have been retrogradely-labelled by HRP uptake from the capillaries in the neurohypophysis (Broadwell and Brightman, 1976). These researchers reported that large quantities (50mg.) of HRP, injected intravenously in the mouse, resulted in extensive labelling of neurosecretory neurones in the hypothalamus as well as numerous brain stem nuclei.

Thus, HRP can circumvent the blood-brain barrier by passing through the permeable capillaries in the median eminence and other circumventricular organs. However, it does seem unlikely that peroxidase in ul. quantities injected directly into the brain can enter systemic blood in sufficient concentration to retrogradely label hypothalamic neurosecretory neurones. Two findings in the present study support this. Firstly, zona reticulata injections did not result in the appearance of any labelled cells in the paraventricular nucleus. Secondly, Broadwell and Brightman, 1976, reported that other neurosecretory neurones in the hypothalamus were labelled e.g. the supra-optic nucleus, by intravenous administration of HRP. This nucleus was not labelled in the present study. Thus, it is suggested that neurones in the region of the paraventricular nucleus project directly to the zona compacta region of the SN. The veracity of this finding must be checked by both electrophysiological and autoradiographic techniques.

All zona compacta animals studied (except one) had labelled cells in the dorsal raphe nucleus thus further supporting the possibility of a projection from this nucleus to the SN. It is interesting to note the presence of a cluster of labelled cells in the region of the superior cerebellar peduncle. These cells are in close proximity to the median raphe nucleus and may partly account for the electrophysiological responses evoked in SN neurones by electrical stimulation in that region (Dray et al., 1976). It is also apparent that the location of these labelled cells is near the ascending noradrenergic axons (Ungerstedt, 1971) and also close to brain region known to

support intracranial self stimulation (Clavier and Corcoran, 1976). It is possible that electrical stimulation of these neurones may influence the firing rate of neurones in the zona compacta region and thus link this ICSS system with the postulated dopaminergic one (Crow, 1972). Again it is essential that further anatomical and electrophysiological studies are performed to confirm these initial observations. This in particular applies to the interstitial nucleus of the stria terminalis, which is known to receive dopaminergic innervation from the zona compacta DA system (Ungerstedt, 1971). Thus, the possibility of a reciprocal connection between zona compacta and the interstitial nucleus of the stria terminalis does exist.

It was observed in the present study that evenly stained axons coursed rostrally from injection sites well-localised to the zona compacta region. These stained axons followed a very similar course to the nigro-striatal pathway (Ungerstedt, 1971(a)) and finally ramified throughout the corpus striatum. This suggests that the SN DA-containing neurones are transporting HRP anterogradely. This form of anterograde transport is clearly different from that reported by Nauta et al., 1975. It is possible that this even staining results from uptake of HRP by normal or more likely damaged neurones (Nauta et al., 1974; Kuypers et al., 1974). In the region of the injection site it was noticed that labelled cells were present which were indistinguishable from retrogradely-labelled cells. This labelling may reflect HRP uptake into damaged neurones or, more likely, from the results of the present experiment, uptake by normal neurones.

The HRP technique, although still in its infancy, has gained

wide acceptance in the neuroanatomical field. However, the present study along with many other recent studies emphasises that the technique is not reliable enough by itself and should be complemented, where possible, by more established anterograde techniques. Although the source of some of the labelled cells indicated in this study may be from HRP uptake by damaged neurones, it is to be hoped that all intact axons terminating in the substantia nigra, close to the injection site, also accumulated HRP to a significant extent and transported it retrogradely to the cell bodies of origin.

The possibility that neuronal cell bodies may be labelled following HRP uptake into either intact terminals or damaged axons makes it essential to further characterise the appearance of these labelled cell types.

In summary, the cells of origin of central fibre systems projecting to the substantia nigra have been investigated using the retrograde horseradish peroxidase (HRP) technique. After HRP injection in the zona reticulata region, HRP reaction granules were found in neuronal cell bodies of the ipsilateral corpus striatum, globus pallidus, dorsal raphe nucleus and frontal cortex. HRP injections, well localised to the zona compacta region of the SN, resulted in the retrograde labelling of cell bodies in the ipsilateral globus pallidus, tail of the striatum, paraventricular nucleus, lateral hypothalamus, interstitial nucleus of the stria terminalis and the region of the superior cerebellar peduncle.

CHAPTER V.

A NEUROANATOMICAL STUDY ON THE STRIATO-NIGRAL PATHWAY IN THE RAT AND BEHAVIOURAL AND BIOCHEMICAL EFFECTS OF LESIONING THIS PATHWAY

INTRODUCTION

It has been established for many years that the corpus striatum projects directly to the SN and the globus pallidus (Papez, 1938; Verhaart, 1950). This projection has recently been intensively studied using the degeneration tracing techniques of Nauta and Gygax (1957) and Fink and Heimer (1967). (Voneida, 1960; Szabo, 1962, 1967, 1969, 1970 and 1972). These studies were all performed in the cat or the monkey and, given the relatively large brain size of these species, it has proved possible to study the topographical distribution of the striatal efferent pathways, both in the globus pallidus and in the SN. All these studies reported that the caudate nucleus and the putamen projected unilaterally to the ventral part of the SN, the zona reticulata region. This projection of the corpus striatum has been confirmed by electron microscope studies which showed that degenerating boutons were present in the SN following lesions in the striatum (Kemp 1970; Grofová and Rinvik, 1970; Hadju, Hassler and Bak, 1973). All these studies reported that the fibres of the striato-nigral pathway formed both axosomatic and axodendritic synapses with the nigral neurones.

There is now good evidence that the striato-nigral pathway is gabaminergic in nature. It has been shown that the SN gamma amino-butyric acid (GABA) concentration decreases by about 50% following either brain hemi-sections at the subthalamic nucleus level or large lesions placed in the ipsilateral striatum (Kim, Bak, Hassler and Okada, 1971). In a further study in the monkey (Kataoka, Bak,

Hassler, Kim and Wagner, 1974) it was reported that the activity of the GABA synthesing enzyme, L-glutamate decarboxylase (GAD) fell dramatically in the ipsilateral SN following transection of the brain at the subthalamic nucleus level. The activity of GAD in the corpus striatum was only slightly reduced 14 days after the transection which suggested that this nucleus contains a large number of GABA-containing neurones that are intrinsic to the striatum. In contrast to the marked GAD changes in SN, the activity of choline acetylase was not significantly altered. This is good evidence that the striato-nigral pathway is not cholinergic in nature. Dopa decarboxylase activity in the SN was severely decreased as would be expected since the transection also interrupted the ascending axons of the nigral DA-containing cells.

In a recent study it has been shown that GAD is unevenly distributed in the SN (Fonnum, Grofova, Rinvik, Storm-Mathisen and Walberg, 1974). Following lesions in the putamen, nucleus caudatus, globus pallidus or the entopeduncular nucleus GAD activity in the SN was markedly decreased and this loss of enzyme was localised and related to the site of termination of the degenerating striato-nigral fibres, within the zona reticulata region. However, these workers also reported decreases in zona compacta GAD activity, especially after globus pallidus and entopeduncular nucleus lesion and suggested that a gabaminergic pallido-nigral pathway may exist. This was in agreement with an earlier study by Hattori, McGeer, Fibiger and McGeer, 1973, which had concluded that the globus pallidus was a source of the GABA-containing terminals in the SN. From the available

biochemical studies on the striato-nigral and pallidal-nigral pathways in the cat (Fonnum et al., 1974) and baboon (Kataoka et al., 1974) it does appear as though the corpus striatum and the putamen are the major sources of the GABA-containing terminals in the SN. However, a recent anatomical study, using the autoradiographic technique of Cowan et al., 1972, showed that both the striatum and the globus pallidus project to the SN (Hattori, Fibiger and McGeer, 1975). The pallido-nigral projection was found to terminate predominantly on DA-containing cells in the zona compacta, whereas the striatum efferents projected largely to the zona reticulata region. This study overcame the previous criticism by Fonnum et al., 1974, which had suggested that the observed pallido-nigral pathway could be a false positive result since globus pallidus lesions would also interrupt striatal efferent fibres.

It has been suggested that the gabaminergic striato-nigral pathway exerts an inhibitory influence on SN DA neurones (Anden and Stock, 1973; Anden, 1974; Kim and Hassler, 1975). Electrophysiological studies have shown that electrical stimulation of the caudate nucleus inhibits the firing of single neurones in the SN and that this effect is blocked by systemic administration of the GABA receptor blocker, picrotoxin (Precht and Yoshida, 1971). Microiontophoretic application of GABA directly onto nigral neurones causes a marked inhibition in their firing rate (Feltz, 1971). This depressant effect of GABA is antagonised by microiontophoretically applied picrotoxin (Crossman, Walker and Woodruff, 1973) and bicuculline methochloride (Dray and Gonye, 1975). These studies failed to distinguish between SN DA neurones and zona reticulata

neurones in terms of either electrical characteristics or drug responsiveness. In a recent study it has been shown that electrophysiologically and pharmacologically identified DA neurones as well as non-dopaminergic zona reticulata neurones are inhibited by GABA applied microiontophoretically (Aghajanian and Bunney, 1973).

The striato-nigral pathway has been reported to be important in the mediation of the depressant effect of systemically administered amphetamine on nigral DA neurones (Bunney and Aghajanian, 1973; 1976). In their more recent paper these workers showed that discrete knife cuts, placed acutely in the crus cerebri region or in the tail of the caudate nucleus abolished the depressant effect of systemically administered d-amphetamine on DA cells located in the ipsilateral SN. Following these lesions the spontaneous firing rate of DA neurones increased and it was suggested that they had been freed from an inhibitory input from the striato-nigral pathway. In their earlier paper Bunney and Aghajanian reported that brain hemitransections, at the level of the subthalamic nucleus, also resulted in an increase in DA cell firing rate. It is possible that this effect could be due to damage to the axons of the DA neurones and this cannot be excluded when considering the effects of the more discrete knife cuts since it was not established whether the DA neurones were damaged or not.

It has also been recently suggested that the striato-nigral pathway may be important for the mediation of the facilitatory effect of neuroleptic drugs on nigral DA cell activity (Kim and Hassler, 1975). These workers reported that intraperitoneal administration of

large doses of haloperidol caused a significant decrease in nigral GABA concentration and it was proposed that this effect may result in the decreased inhibition of dopaminergic nigro-striatal neurones. Although their results do not establish that haloperidol decreased GABA release in the striato-nigral pathway these workers suggested that this was the most likely explanation of their results. This proposal supports the concept of a neuronal feedback mechanism being responsible for increasing nigral DA cell activity, following post-synaptic DA receptor blockade by neuroleptic drugs (Carlsson and Lindqvist, 1963). This concept has gained much support (Anden, Carlsson and Haggendahl, 1969; Horn and Snyder, 1971; Kebabian, Petzgold and Greengard, 1972; York, 1972; Bunney et al., 1973 (a)) but there is no direct evidence that these suggested post-synaptic effects of neuroleptics actually decrease impulse flow in the striato-nigral pathway.

Neuroleptic drugs have been recently reported to block DA receptors located on nigral DA neurones (Groves et al., 1975) but these results conflict with microiontophoretic findings which suggest that these drugs do not have an effect on DA neurones (Aghajanian and Bunney, 1973). However, it is known that iontophoretically applied DA does cause a marked depression of these neurones and that this effect is blocked by chlorpromazine, when administered intravenously but not when applied iontophoretically (Aghajanian and Bunney, 1973). This finding is rather more difficult to explain than the recent report that depression of neurones in the putamen and amygdala by iontophoretic application of DA is antagonised by the iontophoretic application of the neuroleptic, alpha-flupenthixol but not when the

drug is administered intravenously (Ben-Ari and Kelly, 1976). These workers suggest that only by the iontophoretic method is enough neuroleptic administered to overcome the depressant effect of iontophoretically applied DA. However, in the SN the opposite situation exists. One very tentative explanation of the nigral results would be that chlorpromazine, when administered intravenously, causes blockade of post-synaptic DA receptors in the terminal areas of the nigral DA neurones and thereby increase DA cell firing rate by decreasing the impulse traffic in the striato-nigral pathway. Of course, for this to be tenable it is essential that the neuronal feedback mechanism could override the depressant effect of DA. From the iontophoretic data available it does appear that the postulated DA receptors located on DA neurones are distinct from those located post-synaptically in the DA terminal areas.

Although early studies concentrated on the effects of neuroleptics on DA metabolism recent attention has focused on striatal acetylcholine (ACh) metabolism. It is now well established that neuroleptics cause an increase in striatal ACh turnover accompanied by a decrease in ACh concentration (Stadler, Lloyd, Gadea Ciria M and Bartholini, 1973; McGeer, Grenaal and McGeer, 1974; Trabucchi, Cheney, Racagni and Costa, 1974; Guyenet, Agid, Javoy, Beaujouan, Rossier and Glowinski, 1975). This effect of neuroleptics has been shown to be abolished by administration of apomorphine (Stadler et al., 1973; Guyenet et al., 1975). These effects of neuroleptics and apomorphine on striatal ACh metabolism persist following ipsilateral removal of the nigro-neostriatal system (Guyenet et al., 1975) thus providing

evidence that these drug effects are mediated via post-synaptic DA receptors, probably located on ACh neurones in the striatum. The findings that anticholinergic drugs partially block the facilitatory effect of neuroleptics on striatal DA metabolism also suggests that a cholinergic pathway may be involved (O'Keefe, Sharman and Vogt, 1970; Andén, 1972; Andén and Bedard, 1971). This is further supported by recent evidence which demonstrated that degenerating terminals of the nigro-striatal system synapsed with immunohistochemically identified striatal ACh-containing neurones (McGeer, McGeer, Grewaal and Singh, 1975).

The intrinsic organisation of neuronal interconnection in the striatum is poorly understood. Recently it has been proposed that ACh-containing neurones may exert an excitatory effect on striatal GABA-containing cells, the efferents of which form the striato-nigral pathway (Groves et al., 1975). This particular connection has not been demonstrated and the main evidence for this proposal stems from iontophoretic experiments which showed that ACh has a predominantly excitatory effect on striatal neurones (Bloom, Costa and Salmoiraghi, 1965; Spencer and Havlicek, 1974). Following this particular scheme of interconnections i.e. DA cells inhibiting cholinergic cells which in turn excite GABA cells, it follows that DA receptor blockade by neuroleptics would result in an increase in the impulse traffic in the descending striato-nigral pathway. This would be expected to decrease the firing rate of DA neurones in the SN. Obviously this proposed chain of neural connections does not explain the available experimental data. Groves et al., 1975

further suggest, however, that the main site of action of haloperidol is on the DA autoreceptors located on the nigral DA neurones, since intra-nigral microinjections of this drug markedly increase zona compacta cellfiring rate. As discussed in the Introduction to Chapter II this proposal is not consistent with the available microiontophoretic data (Bunney and Aghajanian, 1973).

As an alternative to the DA receptor blockade hypothesis of neuroleptic action, Seeman and Lee, 1974, proposed that these drugs may increase spontaneous DA release by directly promoting membrane fusion between the membrane of the DA granule and the presynaptic membrane. This increase in release was proposed to disinhibit the enzyme tyrosine hydroxylase, present in the DA terminals, by the removal of end product inhibition. This would result in an increase in the synthesis and turnover of DA. This intraneuronal feedback mechanism was largely based on labelled DA release from striatal synaptosome preparations. In this scheme the neuroleptic drugs were suggested to affect DA terminals by a mechanism that did not involve DA receptors. Instead they acted either by promoting membrane fluidisation of the DA granules and the presynaptic membrane, thus enhancing membrane fusion and transmitter release. Alternatively it was proposed that they may displace membrane-bound Ca^{++} ions, thereby facilitating release of transmitter. These mechanisms of neuroleptic action on DA terminals are extremely difficult to distinguish from that proposed to act via DA receptors located on the DA terminals as suggested by a number of workers (Farnebo and Hamberger, 1970, 1971; Kehr et al., 1972; Roth, Walters and

Aghajanian, 1973; Walters and Roth, 1974; Roth, Walters, Murrin and Morgenroth, 1975) and discussed in the Introduction to Chapter II.

The possible multiple sites of action of neuroleptic drugs makes it difficult to investigate whether the predominant effect on DA metabolism and/or DA cell firing rate is mediated via DA receptor blockade on DA neurones or via post-synaptic DA receptors that initiate a concerted neuronal feedback activation of DA neurones. In a bid to separate the presynaptic site of action from the post-synaptic site it was decided in this present study to try and trace the striato-nigral pathway; the most likely neuronal system to mediate the effects of neuroleptics on SN DA cell activity, and lesion it whilst completely sparing the nigro-striatal DA system. The effect of lesioning the striato-nigral pathway, both on normal and neuroleptic-enhanced striatal DA metabolism is described.

METHODS

Autoradiographic Tracing of the Efferent Connections of the Corpus Striatum

The technique used was similar to the autoradiographic method described in detail in Chapter III.

In the experiments described L(4-5,^{3}H) leucine (20-25 µCi/ul.) was injected into the corpus striatum of the male Wistar rat, 200 - 220 gm. Each animal was anaesthetised by a 2% Halothane/air mixture and secured firmly, using blunt ear-bars, in a David Kopf stereotaxic instrument. The labelled solution was delivered through a 30 gauge needle (Pharmaceutical Mfg.Co.) which was attached to a 1 ul. Hamilton syringe and placed stereotaxically into the appropriate area of the corpus striatum. In all cases 0.5 µl. of the labelled solution was injected over a period of 40 minutes and the needle was held in position for a further 20 mins. Labelled leucine was introduced into both the dorsal and the ventral areas of the corpus striatum. The coordinates used were as follows:

	Dorsal placement	Ventral placement
Anterior	0.5mm	0.5mm
Lateral	3.0mm	3.7mm
Vertical	4.0mm	7.3mm

The bregma suture was used throughout as the stereotaxic reference point for the anterior and lateral coordinates and the vertical readings are taken from the cortical surface overlying the injection site.

Following post-injection times of 3-4 days the animals were deeply anaesthetised with chloral hydrate (400 mg./kg. ip.) and perfused through the ascending aorta with 80 mls. of 0.1 M phosphate buffer (pH 7.2), followed by 200 ml. of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Throughout the course of the perfusion the animals were kept on ice.

The subsequent tissue dissection, histological treatment and autoradiographic procedures were identical to those already described in the methods section of Chapter III.

Electrocoagulation Procedures

Adult, male Wistar rats, weighing 190-210 gm. were anaesthetised with a 3% halothane/air mixture and their heads were firmly fixed in a stereotaxic instrument (David Kopf) and anaesthesia continued by a 1% halothane/air mixture. A 001 insect pin with a tip diameter of 0.3mm and insulated to within 0.3mm of the tip by Inslx E-33 resin (Inslx Corp.), was stereotaxically aimed for the ventro-medial part of the crus cerebri. This area contained the fibres of the striato-nigral pathway. Coordinates for the electrode placements were as follows:

Posterior	3.8mm
Lateral	2.4mm
Vertical	7.6-7.7mm

The bregma suture was used as the stereotaxic reference point for the anterior-posterior and the lateral coordinates. The cortical

surface, overlying the lesion site was used as the reference surface for the vertical reading.

Electrolytic lesioning of this region of the crus cerebri was performed with a 12 V DC power supply by passing a current of 200-300 μA for 20-30 seconds so that a total of 6mcoulombs of charge was passed in each case. Following the lesioning procedure, antibacterial agent (Polybactrin) was applied to the operated area and the scalp incision was carefully sutured and the animal allowed to recover consciousness. A period of 1 week elapsed before the rats were subjected to further experimental procedures.

Method of Recording Rotational Behaviour

After recovery from surgical procedures the lesioned rats were tested for their ability to display rotational behaviour, (Ungerstedt and Arbuthnott, 1970), when injected intraperitoneally with either apomorphine hydrochloride (0.5-5 mg./kg.) or d- amphetamine sulphate (2mg./kg.). The rats, following injection, were placed in a spherically shaped plastic bowl and allowed to move around freely. Each complete turn of the animal, occurring during the following 30 minute post-injection period, was recorded by an observer. Notes were also taken of the animals behaviour.

Estimation of Dopamine Concentration in the Corpus Striatum

Dopamine concentrations were estimated using a sensitive, radiometric assay described by Palkovits (1974).

1. Dissection and preparation of striatal tissue

Experimental rats were killed by stunning and decapitation. Their brains were immediately removed, placed on a chilled dissecting plate ($0^{\circ}C$) and both striata were carefully dissected out following removal of the nigral tissue (described later). Individual striata were wrapped in labelled aluminium foil and frozen in liquid nitrogen until required for biochemical assay.

The frozen tissue was homogenised in 0.1 N perchloric acid (PCA) (300 µl. 0.1 N PCA per 10 mg. striatal tissue). The homogenates were then centrifuged at 10,000 g for 15 mins. Since the approximate weight of a single striatum was 40-60 mg. the volume of supernatant obtained following centrifugation was usually 1.2-1.4 mls. From this volume of supernatant it was possible, by taking aliquots, to simultaneously estimate the concentrations of DA, NA, the acidic DA metabolites, homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC), and GABA. (See later)

2. Assay for DA.

300ul. of the supernatant fluid from the striatal tissue homogenates was placed in 15 ml. glass-stoppered centrifuge tubes. Blanks consisted of 300µl. of 0.1 N PCA. In separate tubes standards consisting of 25mg. (free base) of DA and NA in 10µl. of 0.1 N PCA were added to 300µl. of brain extract. The reaction was initiated with the addition of a mixture containing: 500µg. of dithiothreital; 0.5 µmol. of $MgCl_2$; 140µmol. of tris-HCl buffer, pH 9.6; 2.5 µl. of catechol-O-methyl transferase (rat liver enzyme purified according

to the method described by Axelrod and Tomchick, 1958); and 2.5μCi of (^{3}H- methyl) S- adenosyl-methionine. The reaction mixture was incubated for 60 mins. at 37°C and was stopped by the addition of 500 μl. of 0,5Mborate buffer (pH10).

After the addition of non-radioactive carriers (7μg. methoxytyramine, 3μg. of normetanephrine, 3μg of metanephrine) and 1mg. EDTA, the O-methylated products were extracted into 9ml. of water-saturated ethyl acetate-methanol (10:1 v/v) by shaking for 30 secs. The phases were separated by centrifugation at low speed and 8.5 ml. of the organic phase was transferred to another tube containing 0.5ml. 0.5M borate buffer (pH10). 8ml. of the organic phase was then shaken with 0.5 ml. of 0.1 N HCl for 30 secs. The phases were separated by low speed centrifugation and the organic phase was aspirated and discarded. The acid phase was washed with 8ml. of water saturated ethyl acetate, and the organic phase was discarded.

To separate normetanephrine from methoxytyramine, the side chain of normetanephrine was cleaved at the β-hydroxyl group. At timed intervals, 50μl. of freshly prepared 3% (w/v) sodium metaperiodate and then 3 min. later, 50 μl. of 10% (v/v) glycerol were added to each tube. The ^{3}H- methyl vanillin derived from the cleavage of normetanephrine was extracted into 10ml. of toluene; and 9 ml. of the organic phase was transferred to another tube containing 1ml. of 1N NaOH. The ^{3}H- methyl vanillin in this toluene phase was used for the estimation of NA but these results were not included in this study so further details are not included. The aqueous phase from

the periodate cleavage reaction was used for the DA determination. 5ml. of toluene were added to the tubes containing this aqueous phase. Following shaking and centrifugation, 0.5ml of 1M borate buffer (pH 11) and 6 ml. of toluene: isoamyl alcohol (3:2 v/v) were added to each and the ^{3}H-methyl methoxytyramine was counted in 10ml. of NE260 scintillant (Nucleur Enterprises).

Estimation of 3,4 dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the Corpus Striatum by Gas-liquid chromatography and Electron Capture Detection.

The method was based largely on that described by Pearson, and Sharman 1975(a) but included a number of modifications in the extraction procedure (Nicolaou N. unpublished work).

1. Simultaneous estimation of HVA and DOPAC concentrations in the corpus striatum.

In all estimations of these DA metabolites 675 µl. of the supernatant of the striatal tissue homogenates, prepared as described above, was used. This volume of supernatant was transferred to an Eppendorf tube and 0,5ml. of toluene was added. Following mixing for 30 secs. the tube was centrifuged at low speed. The organic phase was discarded and 0.5 ml. of ethyl acetate (Reeve Angel Scientific Ltd.) was added and the tube shaken for 1 minute. Following mixing, the tube was centrifuged for 3 mins. and the ethyl acetate layer was transferred to a reaction vial. This ethyl acetate extraction procedure was performed three times for each sample. In all instances the combined ethyl acetate sample extracts were evaporated to dryness under a stream of N_2.

In the derivatisation step, 0.2 ml. of twice redistilled trifluoroacetic anhydride (Aldrich Chemical Co.Inc.) and 0.1 ml. of redistilled hexafluoroisopropanol (BDH), containing 5% v/v freshly prepared boron trifluoride etherate, were added to the dried residue and reacted at 100°C for 1 hour. Following this step the reaction vial was allowed to cool to room temperature before opening and then evaporating the contents just to dryness at room temperature under a stream of dry N_2 the oily residue was dissolved in 1 ml. of dry ethyl acetate, containing 100 ng. of pentafluorophenyl benzoate, used as an internal standard. 2 µl. of this solution was injected into the gas chromatograph.

2. Gas-liquid chromatography

This was carried out using a Hewlett-Packard model 5710A Gas Chromatograph fitted with Ni electron capture detectors, maintained at a temperature of 250°C. The carrier gas was argon containing 5% methane. It was delivered at a flow rate of 50ml./min. which corresponded to a gas pressure of 40 pounds/sq.in. (psi.). The chromograph column consisted of a 2% SE 52 liquid phase coated on Chromosorb Q (Hewlett Packard) used at an oven temperature of 115°C.

The relative retention times of the trifluoroacetic anhydride and hexafluoroisopropanol derivatives of DOPAC and HVA, with respect to the internal standard, were 0.35 and 0.52 respectively. The areas of the DOPAC, HVA and hexafluoroisopropanol peaks were measured in each case and the ratio of metabolite peak area to internal standard peak area was calculated. These ratios were compared with those in a

standard curve and the amounts of HVA and DOPAC in each sample was calculated. With this method the recovery of both HVA and DOPAC was 90% and the values given are uncorrected for recovery.

Estimation of gamma-aminobutyric acid (GABA) in the corpus striatum and SN using gas-liquid chromatography and electron capture detection

The estimation of GABA concentration was performed in both the SN and the corpus striatum using the method described recently by Pearson and Sharman, 1975(b).

The estimation of GABA concentration in the striatum was performed on a 25µl. sample of the supernatant of the striatal tissue homogenate, the remainder of which was used in the estimation of DA, HVA and DOPAC as previously described. KCl in slight excess of saturation was added to the sample.

1. Dissection and extraction of substantia nigra tissue

In order to dissect out the substantia nigra region, coronal knife cuts were placed at the level of the mammillary bodies and the caudal region of the interpeduncular nucleus. Thereafter, the resulting brain slice was cut down the middle in order to separate the left and right sides. The overlying cortical tissue was carefully removed and a further knife cut, placed just ventral and parallel to the medial lemniscus, defined the dorsal extent of the nigral sample. Thus, the dissected tissue included a section of the crus cerebri interpeduncular nucleus as well as the entire SN. The dissection procedure was such that the nigra ipsilateral to a lesion

was removed first on the same number of occasions as the contralateral nigra in a given batch of animals. The entire dissection occupied between 2-3.5 minutes and the tissue was immediately placed into liquid N_2 and kept there until required for biochemical analysis. At this stage in the dissection the section of brain containing the lesion site was removed for later histological examination before commencing the striatal dissection.

The nigral tissue was homogenised in 700µl. of 0.1 HCl. 25µl. of 12 M PCA were added to this homogenate along with crystalline KCl, in slight excess of saturation. The sample was then centrifuged at a low speed for 5 min. at room temperature. 25µl. of this sample was used for the estimation of GABA.

2. Preparation of chromatographic columns

Amberlite CG 1200 (100-200 mesh) was first cleaned by stirring in 2M HCl and washing again several times with water. This procedure was carried out 3 times. The washed CG 1200 resin was then poured, to a depth of 2 cm., into a Pasteur pipette fitted with a sintered glass filter at one end to retain the resin. Prior to application of brain extracts to the column, 2 ml. 2N NH OH solution was passed through the resin followed by 3 ml. water. Then 2ml. 1M HCl was passed through the column and the resin was finally washed with 10ml distilled water.

1 ml. of 1N HCl was added to the 25 µl. samples of the striatal and nigral extracts, mixed and 0.5 ml. of the mixture was allowed to flow through the CG 1200 column, prepared as above, under gravity.

The column was then washed with 10 ml. of water and the GABA was eluted with 2ml of 2M NH_4OH solution directly into a reaction vial. The sample was then evaporated to dryness in a vacuum desiccator over phosphorus pentoxide (overnight).

3. Formation of the GABA derivative for gas-liquid chromatographic analysis

The dry residue was dissolved in 0.2 ml. trifluoroacetic anhydride and 0.1 hexafluoroisopropanol was added. The reaction vial was tightly closed and left for 1 hour at room temperature. The contents of the vial were then evaporated just to dryness under a stream of dry nitrogen at room temperature. 1 ml. of dry ethyl acetate was then added to the vial which was then closed and shaken. 2ul. of the solution was injected into the gas chromatograph.

For each run of samples, a standard curve for GABA was prepared, using the peak heights derived from known amounts of GABA derivatised as outlined above. This allowed changes in the sensitivity of detection to be monitored and partly offset the disadvantage of not including an internal standard.

4. Gas-liquid chromatography

The gas-liquid chromatographic conditions were the same as those described for the estimation of HVA and DOPAC except that the carrier gas flow rate was 40ml./min. corresponding to a pressure of 30psi, and the column temperature was 92°C. This method had a GABA recovery of 60% and the values given are uncorrected for this recovery.

RESULTS

Autoradiographic Tracing of the Striato-nigral Pathway

The distribution of labelled protein was studied autoradiographically in the brain tissue of 8 rats which had received discrete microinjections of high specific activity tritiated leucine in the region of the corpus striatum. 4 animals were found to have well localised injections in the dorsal region of the striatum and the remaining 4 rats had injections localised at more ventral sites in the striatum. In all the sections studied the background activity was found to be very low thus permitting a clear interpretation of the results.

All the brains injected in the dorsal region of the striatum showed a very similar grain distribution at a post-injection time of 4 days. A summary diagram of this distribution is shown in Fig.51. All the injection sites were localised entirely within the striatum; the fibres of corpus collosum acting as an effective barrier to diffusion of the label. A typical injection site is shown in Fig.52(A). Moderately heavy labelling was present in the globus pallidus and this probably arose from labelled striato-nigral fibres and not from diffusion of label. The injection site was rostral to the globus pallidus and it is clear from Fig.51 that striatal tissue adjacent to the labelled globus pallidus was free from silver grains.

Labelled fibres could be clearly observed coursing ventrally in the internal capsule, with a few directed laterally towards the

globus pallidus. Rostrally, the internal capsule was very heavily labelled but in more caudal planes of section the label was found only in the ventral region of this massive myelinated bundle. Heavy labelling was observed overlying the entopeduncular nucleus but it is not clear whether this was terminal or not. The bundle of labelled fibres, coursing caudally in the internal capsule, constituted the only observed output from the dorsal striatum, apart from the short striato-pallidal pathway. This bundle continued caudally in the crus cerebri. From this tract labelled fibres were seen to course dorsally and enter the substantia nigra. The major terminal region of these fibres was within the ventro-medial aspect of the zona reticulata region, Fig.52(B), with only very light labelling observed in the more lateral areas of the SN or in the zona compacta. The labelling rapidly diminished in planes of section caudal to those shown in Fig.51 and no label was detected in the SN caudal to the emergence of the oculomotor fibres from the brain. No labelling could be observed in caudal areas outwith the SN.

The ventral striatal microinjection of tritiated leucine was deliberately placed in the anterior region of the striatum so as to avoid undue diffusion of label to the globus pallidus. These injections were well localised within the striatum with only very slight labelling in the area immediately ventral to the striatum. All 4 brains injected in this region showed a similar grain distribution at a post-injection time of 3-4 days. The distribution from a single brain is shown in Fig.53. Again the label was found in the globus pallidus (not shown) but it could not be established

whether this originated from labelled terminals or from fibres of passage which were seen to eventually enter the internal capsule via a ventral route. These labelled fibres gathered into a relatively discrete bundle which coursed caudally within the ventro-medio-lateral region of the internal capsule. These fibres contined into the crus cerebri and occupied a more lateral position than that occupied by labelled fibres following a dorsal striatal injection. These fibres finally coursed dorsally and entered the substantia nigra. In this case the pattern of labelling observed in the nigra was topographically distinct from that observed following dorsal striatal microinjections of leucine. The label was largely localised to the lateral region of the zona reticulata with a moderate amount of label overlying a small lateral part of the zona compacta. In the posterior region of the SN the entire zona reticulata region was found to be labelled. No label was observed in planes of section that were caudal to the SN.

The significance of this study, apart from showing the topographical distribution of striato-nigral fibres, was that it permitted the entire course of these fibres to be accurately plotted. At the level of the caudal hypothalamus and the mammillary bodies the plotted pathway was observed to occupy a more lateral course than that reported for the ascending nigro-striatal bundle (Ungerstedt, 1971(a); Palkovits and Jacobitz, 1974).

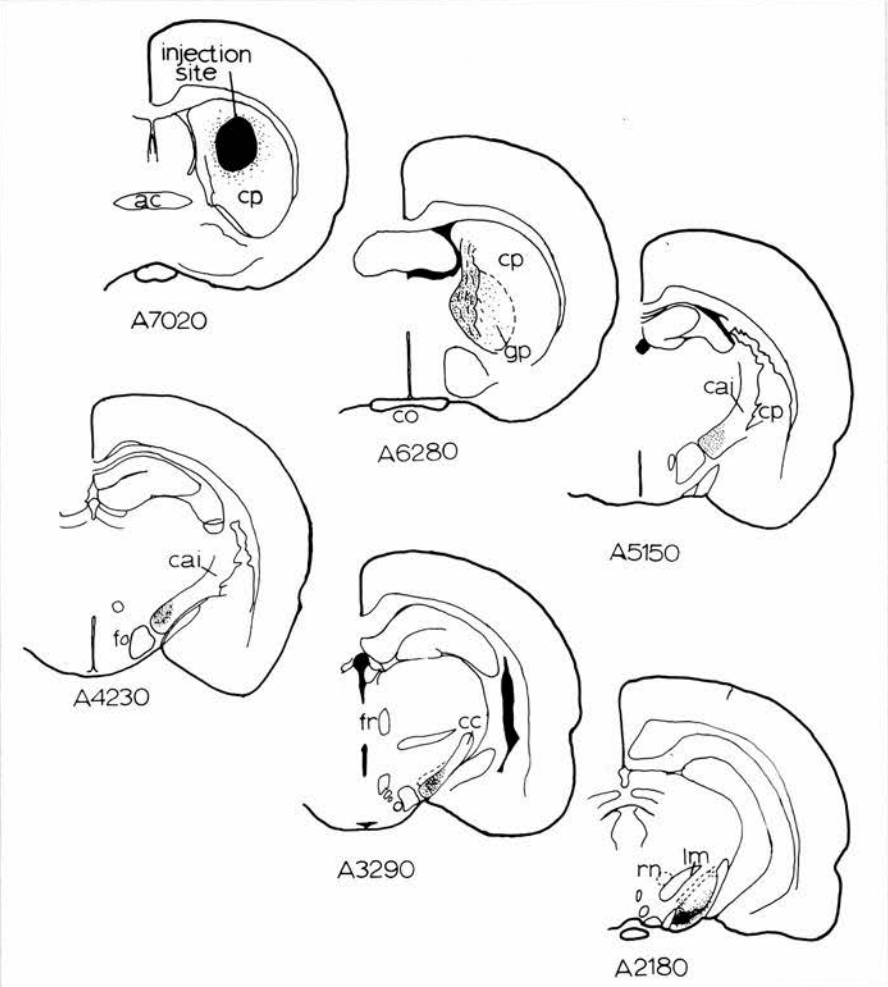

Fig.51

Summary diagram of the silver grain distribution in rat brain following a dorsal striatal injection of L(4,5-^{3}H) leucine. The post-injection survival time was 4 days. These data were summarised from autoradiographs prepared from 4 brains which had been injected in an almost identical site to that shown and projected onto the appropriate coronal plane of section taken from the atlas of König and Klippel (indicated by number under each section).

Abbreviations: ac, anterior commissure; cai, internal capsule; cc, crus cerebri; cp, caudatoputamen; f, fornix; fr, fasciculus retroflexus; gp, globus pallidus; lm, lemniscus medialis; rn, red nucleus.

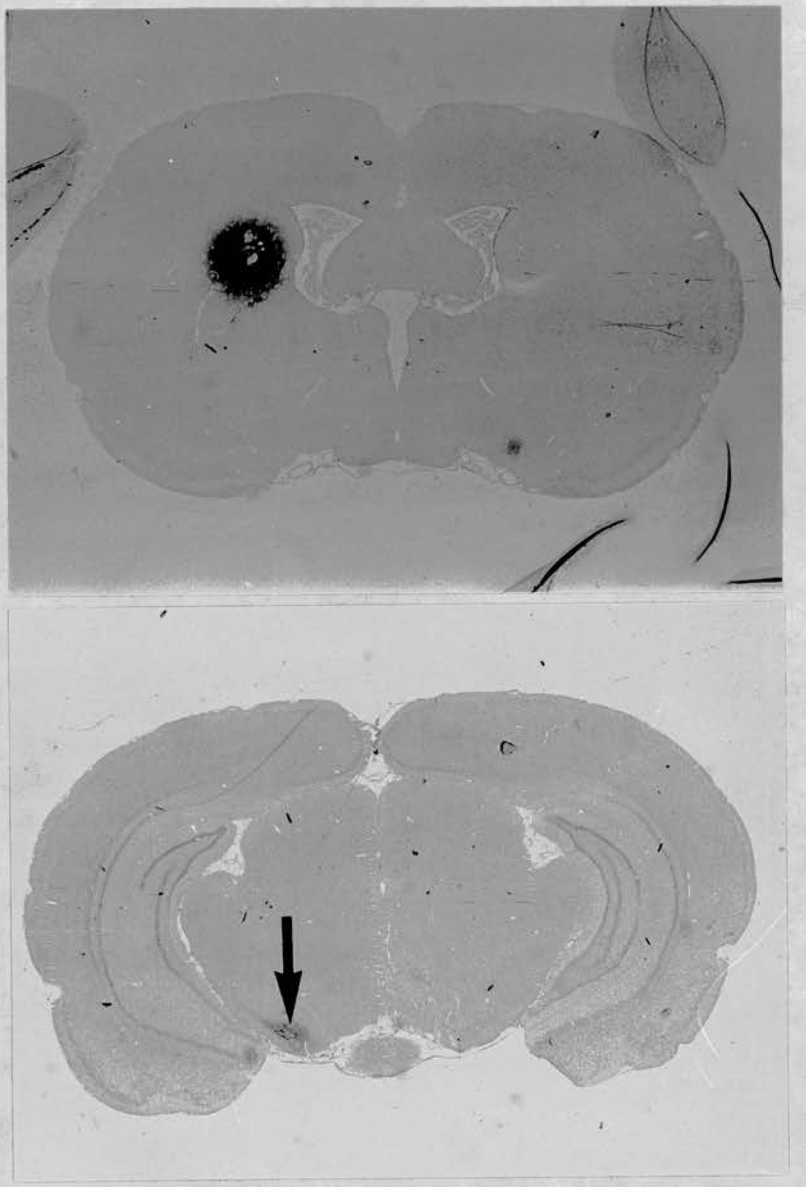

Fig.52(A)

Low power photograph of an autoradiograph of a complete coronal brain section showing the maximum extent of an injection of ^{3}H-leucine into the region of the dorso-rostral region of the striatum.

Fig.52(B)

Low power photograph of an autoradiograph of a coronal section taken from the level of the anterior substantia nigra, showing the site of labelled terminals (arrow) in the medio-ventral zona reticulata region of the substantia nigra, following an injection of ^{3}H-leucine into the ipsilateral corpus striatum (shown above). The post-injection survival time was 4 days. Both sections were stained with haemotoxylin and eosin and the low contrast in the photographs is due to the autoradiographic film being stained with eosin.

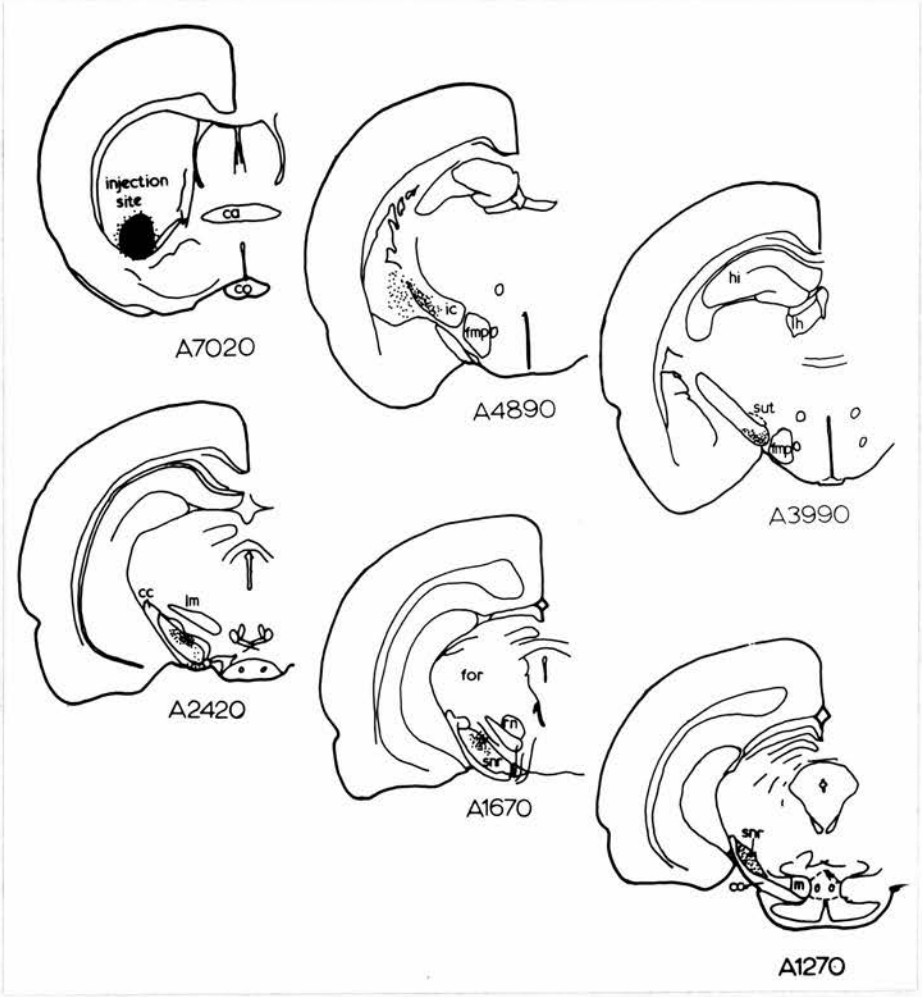

Fig.53

Diagram of the silver grain distribution in a rat brain (black dots) following a ventral striatal injection of 0.5 µl. L(4,5-^{3}H) leucine. The post-injection survival time was 4 days. The grain distribution was that observed in autoradiographs prepared from the brain of a single animal and plotted onto planes of section taken from the atlas of König and Klippel (indicated by number under each section). Abbreviations: ca, anterior commissure; co, optic chiasma; cc, crus cerebri; fmp, fasciculus medialis prosencephali; for, midbrain reticular formation; hi, hippocampus; ic, internal capsule; lh, lateral habenular nucleus; lm, lemniscus medialis; snr, substantia nigra, zona reticulata; sut, subthalamic nucleus.

Effect of Discrete Electrolytic Lesions Placed Unilaterally in the Region of the Striato-nigral Pathway

1. Behaviour Studies

A period of 1 week was the minimum time after lesioning at which the animals were tested behaviourally. The animals were placed in a rotometer (Ungerstedt and Arbuthnott, 1970) and tested for their ability to display rotational behaviour when treated with either apomorphine, in a dose range 0.5- 5mg./kg. or d-amphetamine 2mg./kg. All animals with lesion sites well localised to the ventral crus cerebri or zona reticulata region showed a relatively intense rotational behaviour commencing 1-2 minutes after apomorphine administration. Lesion sites from an initial series of 8 turning animals is shown in Fig.54. Fig.55(B) shows the extent and position of a typical discrete electrolytic lesion from a turning rat and this lesion site was in the region of the plotted striato-nigral pathway fibres (Fig.55(A)). When treated with apomorphine these lesioned animals showed a marked asymmetric front limb posture, with the leg contralateral to the lesion crossing the other one. This resulted in the rats turning towards the lesioned side. A dose response curve for apomorphine-induced turning behaviour in these animals is shown in Fig.56. The threshold dose of apomorphine required to elicit turning was found to be approximately 0.5 mg./kg. and turning increased markedly in response to doses up to 2 mg./kg. Thereafter, there was a sharp decline in the rate of increase in turning with increasing apomorphine dosage. In this study 5 mg./kg. was the highest dose of apomorphine administered. D-amphetamine, 2mg./kg.,

also caused turning behaviour towards the lesioned side. However, in this instance a dose-response curve was not plotted but at this dosage of d-amphetamine a group a 6 lesioned rats were found to turn 184±54 (Mean + S.D.) in the 30 minute period following injection.

It was observed that the striato-nigral lesioned animals did not behave abnormally when they were not treated with drugs. There was no obvious motor dysfunction and the animals did not spontaneously turn to any significant extent, when placed in the rotometer without prior drug treatment. In the subsequent biochemical experiments striato-nigral lesioned animals which turned more than 100 turns/30 minutes in response to 2mg./kg. apomorphine are termed "lesioned animals".

Another group of 8 rats with lesion sites shown in Fig.57 were also tested for apomorphine induced turning behaviour. In all these animals apomorphine (1-5mg./kg. 1.p.) failed to cause turning either towards or away from the lesioned side. None of these lesions interrupted the ascending fibres of the DA neurones as concluded from the histological results and the ipsilateral striatal DA estimations. The concentration of DA in the striata of 4 control, unlesioned animals was 8.4±2.01 µg./gm. tissue and this estimate was not significantly different from that in striata, ipsilateral to the lesion, obtained from 8 non-turning, lesioned animals; 8.31±2.14 µg./gm.

2. Biochemical Effects of Striato-nigral Lesions

Table 2 shows the concentration of GABA in the striatum and SN

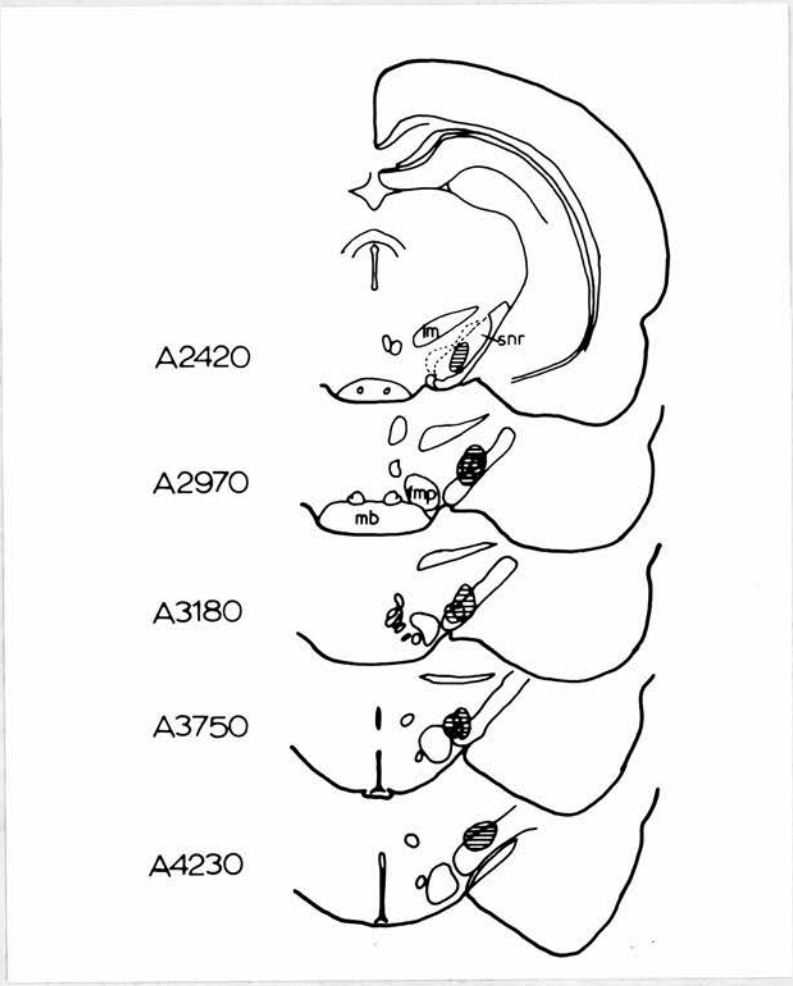

Fig.54

Summary diagram of electrolytic lesion sites from animals which showed apomorphine-induced turning behaviour. Each lesion, indicated by horizontal hatching, resulted from the passage of 6mCoulombs of charge. All sites indicate the maximum extent of the lesion. The numbers of the left refer to the plane of section modified from the atlas of König and Klippel.

Abbreviations: fmp, fasciculus medialis prosencephali; lm, lemniscus medialis; mb, mammillary bodies; snr, substantia nigra, zona reticulata.

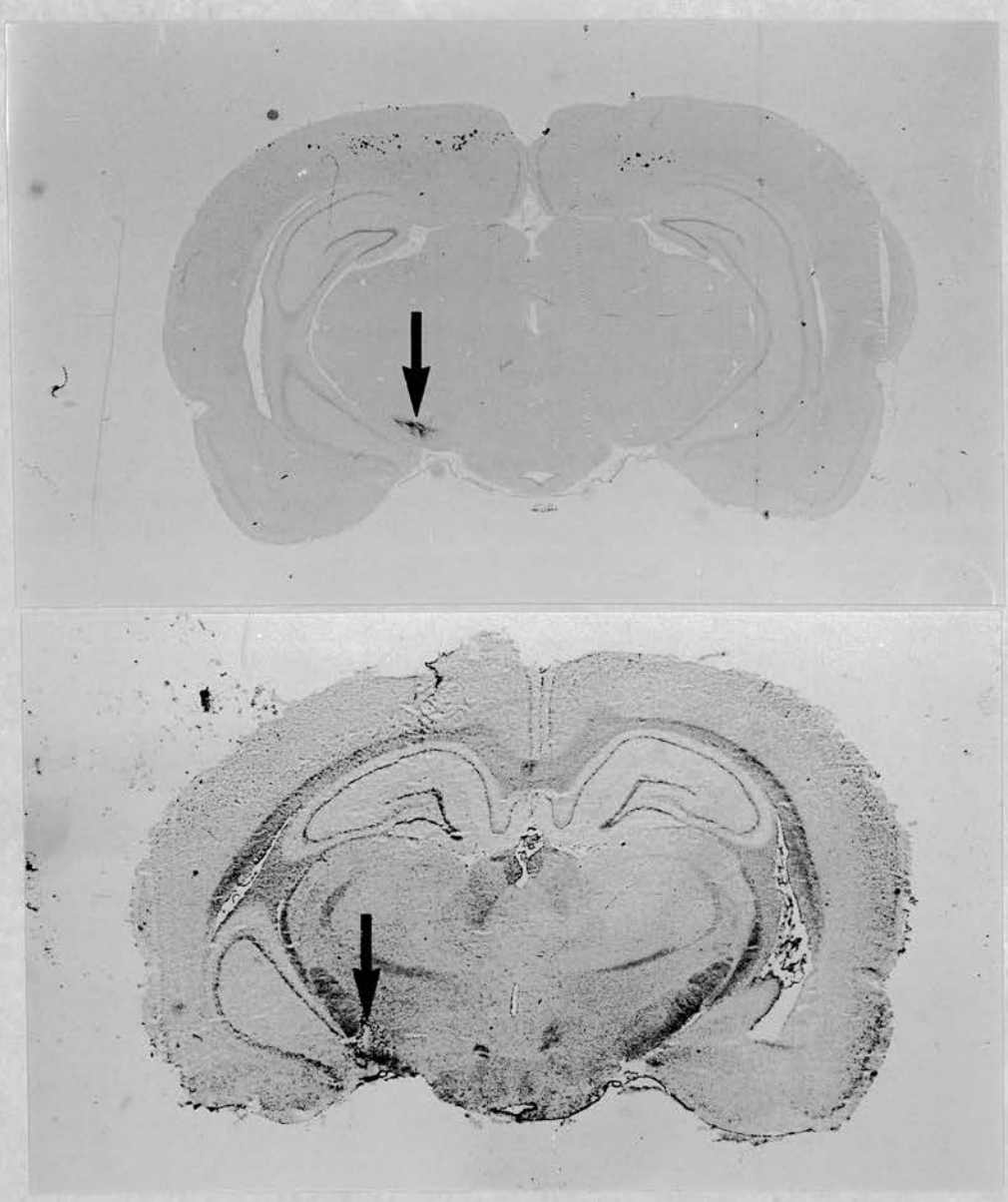

Fig.55(A)

Low power photograph of an autoradiograph of a complete coronal brain section taken from the level of the posterior hypothalamus, showing the location of labelled fibres of the striato-nigral pathway (arrow), following an injection of ^{3}H-leucine into the dorsal region of the ipsilateral striatum. The post-injection survival time was 4 days. Staining: haemotoxylin and eosin.

Fig.55(B)

Low power photograph of a coronal brain section taken from the same brain level as the section shown in Fig.55(A), showing the site of a discrete electrolytic lesion (arrow), well localised to the plotted course of the striato-nigral pathway in the ventral region of crus cerebri. This lesion caused the animal to turn towards the side of the lesion ($>$ 100 turns in 30 minutes) following administration of 2 mg./kg. apomorphine. Staining: Luxol fast blue and cresyl violet.

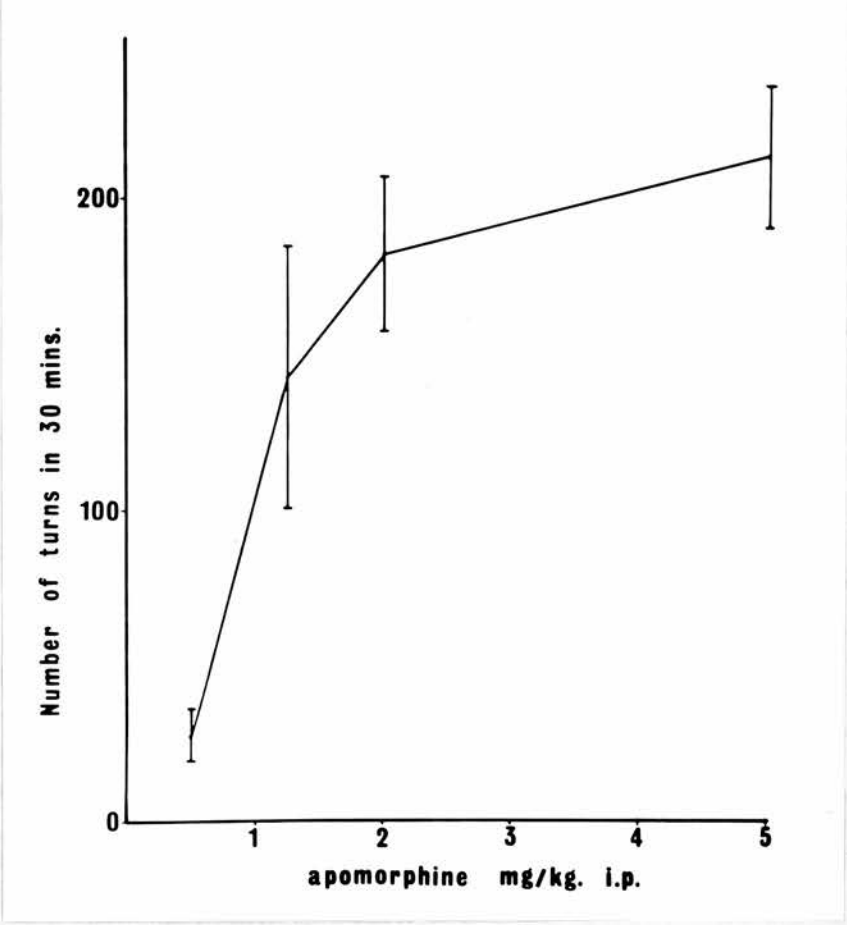

Fig.56

Dose-response curve of apomorphine-induced turning behaviour in rats with lesions in the striato-nigral pathway. The number of turns (ordinate) refers to those counted in a 30 minute period following administration of apomorphine (i.p.) Each point is the mean of at least 8 animals and the bars represent $\pm$ S.D.

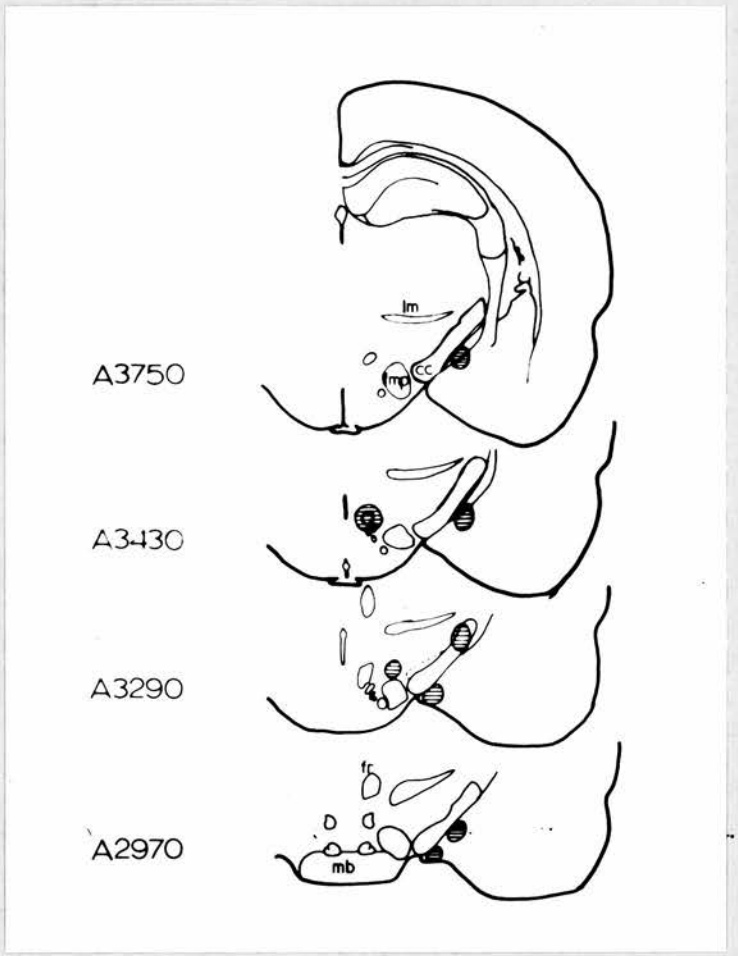

Fig.57

Summary diagram of lesion sites from animals which did not show apomorphine-induced turning behaviour. The lesion details are similar to those described for Fig.54. All lesion sites were projected onto the appropriate plane of section, modified from the atlas of König and Klippel, 1963.

from individual lesioned animals, three weeks after lesioning, It was observed that, between animals, there was quite a large variation in nigral GABA concentration. It is uncertain as to why this was the case but perhaps it reflects the variation in SN dissection time between animals. GABA is also unevenly distributed in the SN (Fonnum et al., 1974) and it could be that small differences in dissection could account for relatively large variations in GABA concentration estimated in the SN of individual animals. To partly overcome this variation between animals it was decided to compare the GABA concentration in the SN ipsilateral to the lesion with that estimated in the contralateral SN of the same animal. In the untreated lesioned group the ipsilateral SN always had a lower concentration of GABA than the contralateral SN. A paired t-test (2-tailed) on the differences between the two sides showed that this fall was significant ($p < 0.005$). In contrast, no significant difference was found between the ipsilateral and contralateral striata in untreated animals. The lesion sites found in these 6 untreated lesioned animals are shown in Fig.58 with one exception due to an inadvertant loss of histological material.

Also included in Table 2 are the striatal and nigral GABA data from haloperidol-treated, lesioned animals referred to in a subsequent biochemical experiment in the last part of this Results section. These animals showed similar changes in GABA concentration in the striatum and SN as those found in the untreated group included in Table 2. There was a significant fall in the GABA concentration in the SN ipsilateral to the lesion ($p < 0.001$: paired t-test, 2-tailed)

but no significant difference between the ipsilateral and contralateral striata ($p > 0.1$: paired t-test). Haloperidol (1mg./kg.) did not have any significant effects on either striatal or nigral GABA concentration when compared with those from untreated lesioned animals. However, it is unlikely that a small effect would be revealed since the variation within groups is quite large. The locations of the lesion sites in these haloperidol-treated animals were plotted from the histological material and a summary diagram of these sites is shown in Fig.59. It can be seen that these lesions, in untreated and treated animals, were well localised to the ventral part of the crus cerebri and only caused minimal damage outside this region. The positions of the lesions were in relatively good agreement with the plotted position of the striato-nigral pathway, although it is unlikely that all the lesions interrupted the entire projection. However, the decrease in GABA on the lesioned side in individual animals was approximately 40-50% and was similar to that reported by Kim et al., 1971, following complete brain hemisections at the subthalamic nucleus level.

3. Striatal DA Metabolism

The effect of striato-nigra pathway lesions on striatal DA metabolism was investigated in a series of 8 lesioned animals. The levels of DA, HVA and DOPAC, in both ipsilateral and contralateral striata, as well as control striata from unlesioned animals are presented in Table 3. It is clear that the lesions did not cause a fall in DA concentration in the striatum, ipsilateral to the lesion, indicating that the nigro-striatal DA system was still intact on that

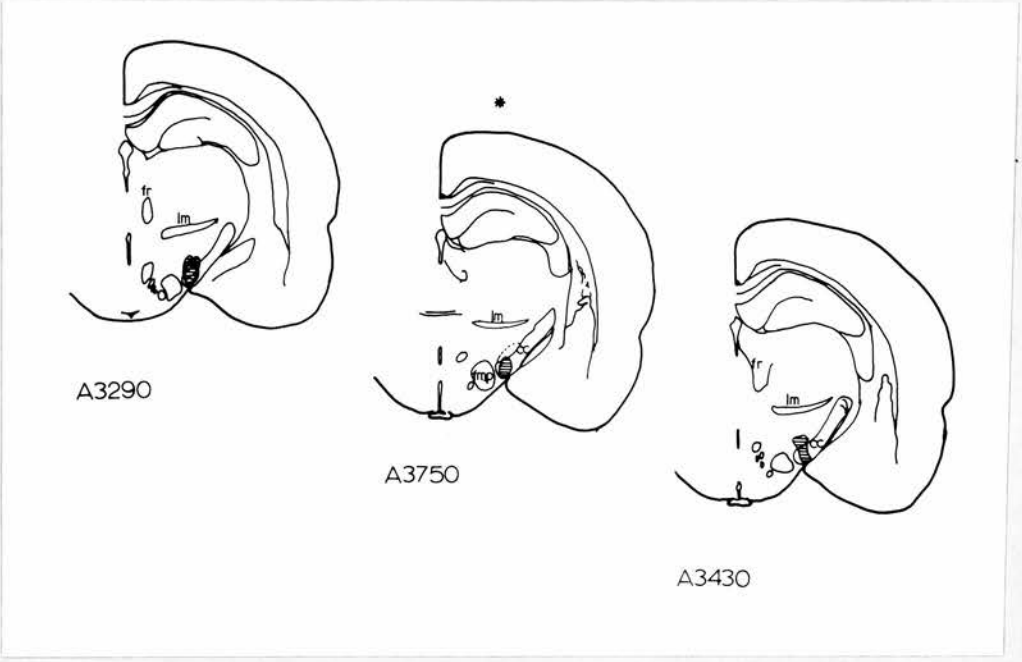

Fig.58

Summary diagram of the lesion sites from turning, untreated animals. The GABA estimations, shown in Table 1, for the untreated lesioned group, were obtained from these animals. The lesion details are the same as those described for Fig.54.

Abbreviations: cc, crus cerebri; fmp, fasciculus medialis prosencephali; fr, fasciculus retroflexus; lm, lemniscus medialis.

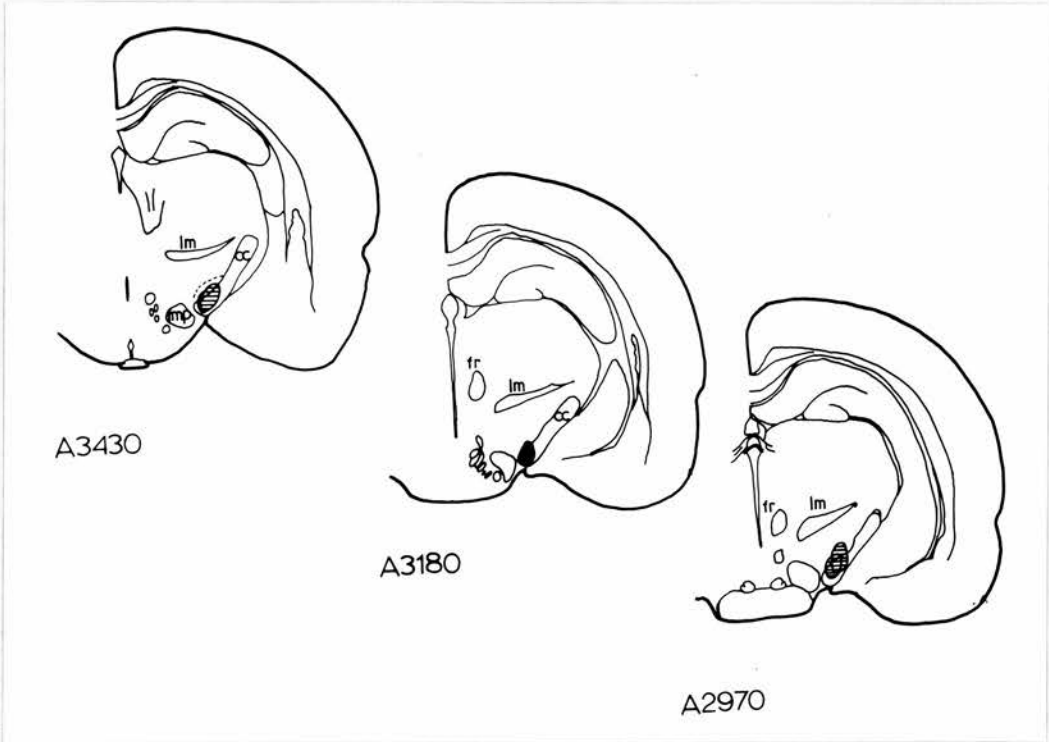

Fig.59

Summary diagram of the electrolytic lesion sites in the group of haloperidol-treated turning animals ($n = 8$). Lesion details are the same as those described for Fig.54. All lesion sites were projected onto the appropriate plane of section modified from the atlas of König and Klippel, 1963 (indicated by the number under each section). Note that in section A 3180 the sites of the lesions were virtually co-extensive as indicated by the complete shading of the area.

Abbreviations: cc, crus cerebri; fr, fasciculus retroflexus; lm, lemniscus medialis.

TABLE 2

GABA concentration in the substantia nigra and corpus striatum of untreated and haloperidol-treated rats with lesions in the striato-nigral pathway. All experimental rats were lesioned on the left side. The animals all displayed turning behaviour towards the lesioned side ($>$ 100 turns/30 min.) when tested with 2 mg./kg. apomorphine, administered intraperitoneally. This test procedure was performed at least 1 week before haloperidol treatment. Results shown are from untreated lesioned animals and from lesioned animals treated with haloperidol (1mg./kg.) given 30 min. before sacrifice. GABA concentration is expressed in µg./gm. wet weight of tissue $\pm$ S.D. P values are from a paired t-test (2-tailed) and refer to the values estimated in the corresponding contralateral brain region.

* $P < 0.005$

** $P < 0.001$

TABLE 2 (for legend, see opposite page)

	Rat Number	Substantia nigra		Corpus striatum	
		Left	Right	Left	Right
Untreated	1	406	525	-	-
	2	335	461	-	-
	3	85	164	159	176
	4	172	394	211	129
	5	135	215	53	88
	6	76	169	69	73
	Mean ± S.D.	201±125*	321±144	123±65	116±40
Haloperidol-treated	1	263	635	74	26
	2	122	312	260	166
	3	86	170	277	229
	4	115	504	178	119
	5	104	162	71	119
	6	176	261	95	265
	7	-	144	65	283
	8	161	360	62	271
	9	151	487	-	-
	10	220	353	-	-
	11	305	442	-	-
	12	293	570	-	-
	Mean± S.D.	181±73**	367±157	135±84	185±86

TABLE 3

Dopamine (DA), homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) concentrations in the corpus striatum of control rats and rats lesioned in the region of the left striato-nigral pathway.

The lesioned animals all displayed turning behaviour towards the lesioned side ($>$ 100/30 min.) when treated with 2mg./kg. apomorphine intraperitoneally. This test procedure was performed at least 7 days prior to sacrifice of the animals. DA, HVA and DOPAC concentrations are expressed as µg./gm. wet weight of tissue $\pm$ S.D. 8 estimations were performed in each group. Both striata were removed from 4 unlesioned rats and the biochemical data from these striata were pooled. There were no significant differences in DA or metabolite concentrations between the intact and lesioned sides of the lesioned rats and these values were not significantly different from those in control striata.

	Lesioned animals		
	Intact side	Lesioned side	Control Striata
DA	9.19$\pm$1.6	9.41$\pm$1.3	8.4$\pm$2.01
HVA	1.40$\pm$0.36	1.19$\pm$0.32	1.10$\pm$0.04
DOPAC	1.20$\pm$0.30	1.05$\pm$0.17	1.15$\pm$0.20

side. Considering the lesioned animals alone there were no significant differences in striatal DA, HVA and DOPAC concentrations between the sides and these values were not significantly different from those estimated in control striata ($p > 0.1$, Student's t-test, 2-tailed).

4. Effect of Haloperidol on DA Metabolism in Control and Striatonigral Lesioned Animals

Haloperidol, 1mg./kg., was administered intraperitoneally to each animal 30 min. prior to sacrifice. As shown in Fig.60 this dose of haloperidol caused a highly significant increase in striatal HVA and DOPAC concentrations in unlesioned animals when these values were compared with values obtained from an untreated control group of animals ($p < 0.001$: Student's t-test, 2-tailed). The striatal DA concentration in haloperidol-treated group of animals did not differ significantly from that measured in striatal tissue obtained from the untreated group. It should be noted, however, that there is an unusually high variance in the striatal DA estimations from the untreated group of animals (see column denoted (a) in Fig.60) caused by 2 inexplicably low estimations. Thus, the striatal DA concentration in these animals is very likely to be artificially low.

In lesioned rats the effect of haloperidol on DA metabolism in the striata ipsilateral to the lesion was compared with that found on DA metabolism in the contralateral striata (Fig.61). The lesion sites and GABA concentrations in the striata and SN of the haloperidol treated animals have previously been referred to and described in the text and are shown in Table 2 and Fig.59 respectively. As shown in

Fig.61, haloperidol caused a highly significant increase in the concentrations of HVA and DOPAC and a highly significant decrease in DA concentration in both the ipsilateral and contralateral striata of lesioned rats when compared to the DA and metabolite concentrations estimated in the striata of a group of untreated, lesioned rats. (In all comparisons, $p < 0.001$: 2-tailed, Student's t-test). There were no significant differences in the haloperidol-induced striatal DA, HVA and DOPAC changes between the lesioned and intact sides of the treated animals.

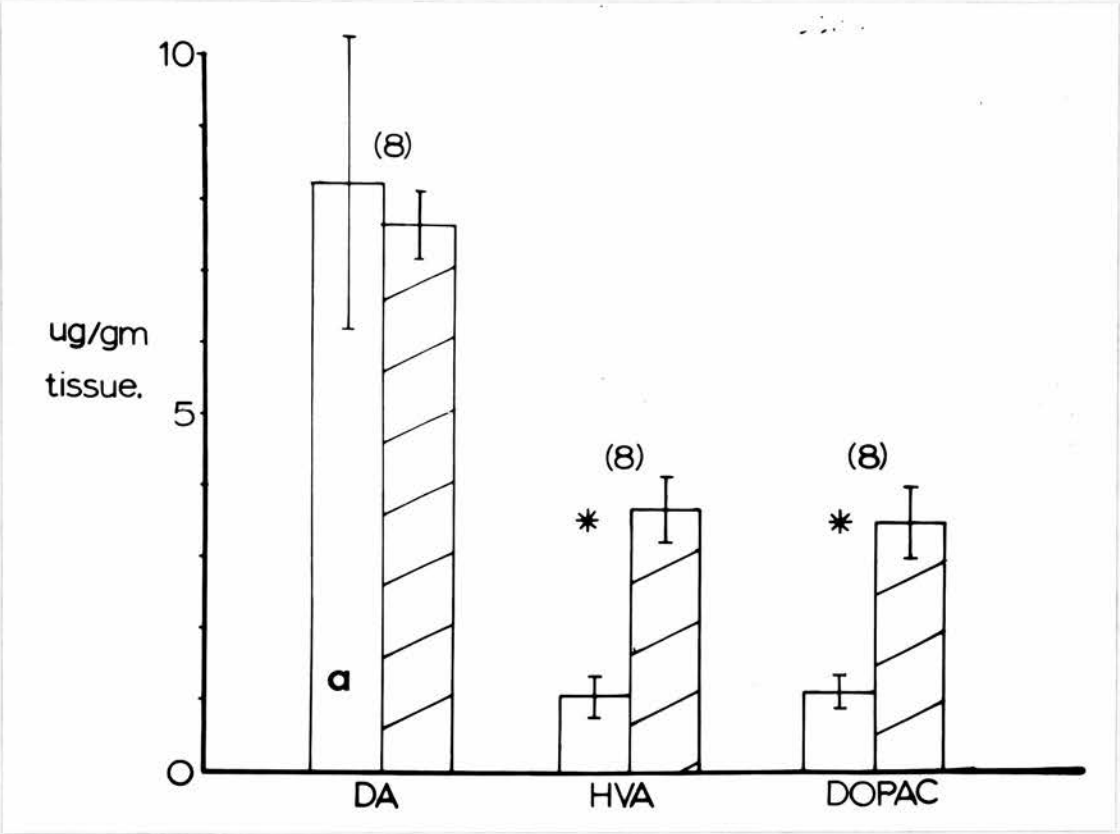

Fig.60

Effect of haloperidol on the concentration of striatal DA, HVA and DOPAC in unlesioned rats. Haloperidol (1 mg./kg.) was administered i.p. 30 minutes before sacrifice. The unhatched columns represent the mean values obtained from non-treated, unlesioned rats and the hatched columns indicate the mean striatal values obtained from haloperidol-treated animals. The bars represent $\pm$ S.D. and the numbers in parentheses indicate the number of estimations in each column.

* Significantly different from untreated animals, $p < 0.001$ (Student's t-test, 2-tailed).

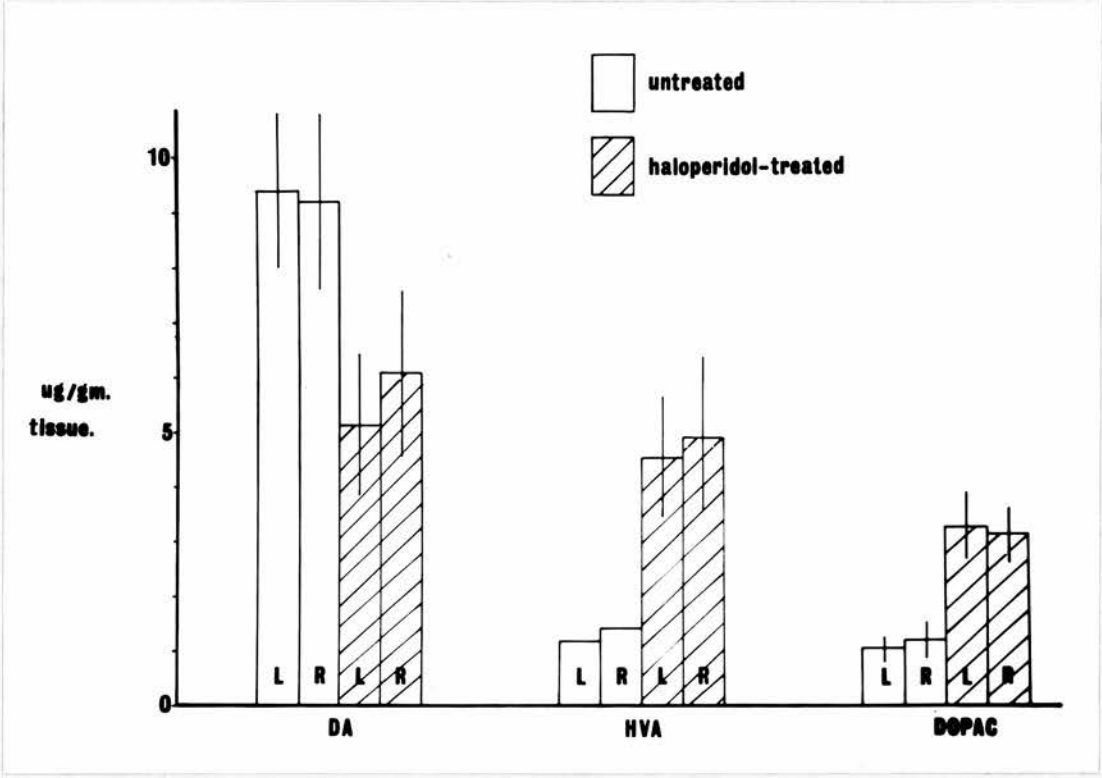

Fig.61

Effect of haloperidol on the concentration of striatal DA, HVA and DOPAC in rats with lesions in the region of the striato-nigral pathway. Haloperidol (1mg./kg.) was administered i.p. 30 minutes before sacrifice. All animals were lesioned on the left side (L) and had previously shown turning behaviour ($>$ 100 turns in 30 mintes) in response to 2 mg./kg. apomorphine. The right side (R) was unlesioned. Both sides of the lesioned, haloperidol-treated group had significantly different concentrations of DA (decrease), HVA and DPOAC (both increased) when compared with striata from the untreated group ($p < 0.001$; Student's t-test, 2-tailed). There were no significant differences in DA, HVA and DOPAC concentrations between the left and right sides of the haloperidol-treated group. Each column is the mean of 8 determinations and the bars represent $\pm$ S.D.

DISCUSSION

The autoradiographic tracing technique proved to be very useful in this present study. Since only the perikarya of neurones in the region of the injection site can incorporate the labelled leucine (Cowan et al., 1972) it can be stated with a fair degree of certainty that the striato-nigra pathway described has its cell bodies of origin in the corpus striatum. An investigation into the topographical distribution of the striatal efferents in the SN was possible but was limited because of the relatively small size of the nucleus and the spread of the injected leucine by diffusion. It is interesting to compare these results with those that have been described for other species in which the topographical relationship between the corpus striatum and SN have been investigated. It has often been reported that the head of the caudate nucleus projects directly to the medial-rostral area of the zona reticulata (Voneida 1960; Szabo, 1962, 1970). There was good agreement in the present investigation with these findings. The dorsal striatal injections were placed in an area of the striatum that corresponded anatomically to the caudate nucleus, although in the rat there is no separation between the caudate nucleus, and the putamen as described in other species. The ventral striatal injections resulted in labelling of the posterior and ventro-lateral areas of the SN. These results were similar to those reported from degeneration experiments involving the putamen of the cat and the monkey (Nauta and Mehler, 1966; Szabo, 1970).

The predominant projection of the striatum in the rat does appear

to be to the ipsilateral zona reticulata region of the SN with only a weak projection to the zona compacta region. In a recent paper Hattori, Fibiger and McGeer, 1975 reported, from electron microscope studies, that the striato-nigral pathway in the rat projected mainly to the zona reticulata region. In an effort to clearly separate the DA-containing cells from the zona reticulata cells in their EM material these workers employed intraventricular injections of 6-OH DA. This agent caused degeneration of the DA neurones in the SN and thus it could be observed whether the terminals of the striato-nigral fibres were synapsing with these histologically distinct degenerating cells or with normal cells located in the zona reticulata. It was established that 84% of the total labelled synapses made contact with normal dendrites or spines while only 3.5% made contact with degenerating dendrites or spines. When the relative areas occupied by the two types of cell were taken into consideration, the relative grain density was about 5 times greater for boutons synapsing with normal dendrites than for those synapsing with degenerating dendrites. However, these results were obtained from leucine injection sites that were largely confined to the dorsal region of the striatum. From the present results it does appear that the ventral region may have a more pronounced projection to the zona compacta region, but it cannot be established whether these fibres actually synapse with DA-containing neurones.

The anatomical results, apart from showing a general topographical distribution of striatal efferents within the SN, also demonstrate that the striato-nigral pathway is well localised to the ventral tip

of the crus cerebri. Although the striatal efferent fibres do occupy this relatively discrete area there does seem to be a topographical distribution of these fibres. In this mapping study it was important that the extent of the striato-nigral pathway was defined so that it was possible to approximately estimate the degree of fibre interruption caused by the discretely placed lesions. Before discussing the lesion results it is pertinent to stress that autoradiographic evidence has been obtained for a gabaminergic pathway from the globus pallidus to the zona compacta that courses in an almost identical pathway to that described in this study (McGeer, Fibiger, Maler, Hattori and McGeer, 1974; Hattori et al., 1975). Therefore it is likely that lesions placed in the ventral crus cerebri would also interrupt this pallido-nigral pathway.

The electrolytic lesions placed in the region of the ventral tip of the crus cerebri completely spared the nigro-striatal fibres as attested by the normal DA levels present in the striatum ipsilateral to the lesion (Table II) but they did decrease GABA concentration in the ipsilateral SN. The variation present in the GABA estimations from the SN of lesioned animals, especially on the unlesioned side may reflect slight differences in the sample of nigra dissected out or in the timing of the dissection. It is well known that a post-mortem rise in brain GABA occurs (Alderman, Schellenberger, 1974; Balcom, Lennox and Meyerhoff, 1975) and in an area like the SN, known to contain the highest GABA concentration of any brain region (Fahn and Cote, 1968; Kanazawa, Miyota, Tokokura and Otsuka, 1973) this may play a part in explaining the variation in these results.

In the present experiments the dissection time for removal of the SN was at least 2 min., by which time the major part of the post-mortem rise in brain GABA is over (Alderman and Schellenberger, 1974), but the post-mortem GABA profile in the SN is unknown and may be different from that reported for whole brain. The estimations of GABA concentration in the SN contralateral to the lesion were intermediate to values reported in the literature. (443 µg/gm. Balcom, Schellenberger, 1974; determined after microwave fixation of the brain. 1060 µg./gm. Kim et al., 1971; time interval from sacrifice until tissue freezing not reported).

It is interesting to note that complete hemisection of the brain between the substantia nigra and the corpus striatum has been reported to decrease SN GABA concentration by between 40-50% (Kim et al., 1971), which is similar to the decrease recorded in individual lesioned animals in this present study. From both these studies there would appear to be GABA in the SN that does not derive from GABA-containing cells in brain areas rostral to the subthalamic nucleus. This "residual" GABA would not appear to be localised in GABA-containing cell bodies in the SN since only few nigral cells show labelling when incubated with H-GABA (Hattori et al., 1973). The non-significant decreases in striatal GABA concentration observed following striato-nigral lesions are in agreement with the hemi-section work reported by Kim et al., 1971. Thus, it would appear that there are GABA systems that are intrinsic to the striatum and that these systems constitute the major source of striatal GABA.

The lesions, described in this study, apart from affecting

nigral GABA concentration, also produced an animal preparation that was very similar to that reported by Swedish workers (Andén, Dahlström, Fuxe and Larsson, 1966; Anden, Rubenson, Fuxe and Hökfelt, 1967) following unilateral removal of the corpus striatum. Both preparations displayed turning behaviour towards the side of the lesion when treated with high doses of apomorphine and amphetamine. These workers suggested that those drugs mediated their effect via DA receptors situated in the remaining striatum. In the unilaterally nigro-striatal lesioned rat preparation described by Ungerstedt, 1971(b) it is quite clear that the doses of apomorphine required to elicit turning are much smaller than those required in the striatal and striato-nigral lesioned animal. Ungerstedt, 1971(c) suggested that this was because of the development of supersensitivity in the striatal DA receptors following removal of the normal dopaminergic innervation. In recent work it has been shown that lesions located in an almost identical position to those reported here, abolish apomorphine-induced turning behaviour in rats lesioned unilaterally in the nigro-striatal system (Ungersted and Marshall, 1976). Thus the integrity of the striato-nigral pathway may be very important for influencing motor behaviour following striatal DA receptor activation by DA agonist drugs. This is supported by the recent finding of a striato-nigral-thalamic pathway (Deniau, Feger and Guyader Le, 1976).

These workers showed that cells in the zona reticulata region of the SN, which were inhibited by electrical stimulation of striatum, also were antidromically invaded following electrical stimulation of the ventro-lateral nucleus of the thalamus.

This may be the pathway by which DA receptor activation in the striatum influences motor behaviour.

The striato-nigral lesioned animals show turning behaviour that is similar to that reported in rats following unilateral lesions of the zona reticulata region (Dray, Oakley and Simmonds, 1975(a); Dray, Fowler, Oakley, Simmonds and Tanner, 1975(b)). Only 1 animal was lesioned in the zona reticulata in the present study and showed turning behaviour. Unilateral elevation of SN GABA concentration by local injection of ethanolamine-O- sulphate (EOS), an irreversible inhibitor of GABA transaminase (GABA-T), also causes rats to turn in a similar manner when treated with high doses of apomorphine (Dray et al., 1975(a)). These workers reported that this elevation of GABA did not occur when EOS was injected at sites above the SN but this data is difficult to reconcile with the recent report by Pycock and Horton, 1976, which showed that microinjection of EOS into the globus pallidus resulted in massive increases in GABA throughout the brain, in the order of 500-700%. Perhaps the most surprising finding following these intra-nigral injections of EOS was that they caused a decrease in the DA content of the ipsilateral striatum and it was suggested that this represented an increase in striatal DA metabolism due to the elevation of GABA inhibiting nigral inhibitory interneurones which synapsed with, and excited the SN DA cells.

In contrast to the results of Dray et al., 1975(a), it has been reported that microinjection of GABA into the SN results in a marked increase in forebrain DA concentration (Andén and Stock, 1973).

This rise was attributed to a direct inhibitory effect of the injected GABA on the DA cells, since a marked rise in striatal DA concentration is known to occur following cessation of impulse flow in the nigro-striatal DA system (Kehr, Carlsson, Lindqvist, Magnusson and Atack, 1972). In a further paper (Andén, 1974) it was shown that systemic administration of amino-oxyacetic acid (AOAA), an inhibitor of GABA-T, resulted in a marked deceleration of the disappearance of striatal DA, following the administration of alpha-methyltyrosine methylester. This again supported the possibility that the SN cells were being inhibited by GABA. Thus, the reported effects on striatal DA metabolism, by the elevation of SN GABA concentration are completely contradictory and perhaps serve as a warning against administering drugs intracerebrally. In particular, it is very difficult to eliminate the possible non-specific depressant or excitatory effects of the injected drugs on the neurones under investigation.

The present study does not support the hypotheses that the striato-nigral pathway exerts either an inhibitory (Bunney and Aghajanian, 1973; Kim and Hassler, 1975) or an excitatory (Dray et al., 1975(a,b)) influence on the nigro-striatal DA system since extensive interruption of the gabaminergic pathway did not result in detectable alterations in DA metabolism in the ipsilateral striatum. In these experiments it was very important to check the integrity of the nigro-striatal DA system because it has been reported that partial lesioning of this system results in a marked increase in striatal DA synthesis (Glowinski, Agid and Javoy, 1973), presumably due to a

biochemical hyperactivity in the remaining DA neurones. The failure to detect changes in striatal DA metabolism, following striato-nigral pathway lesions strongly suggests that this pathway does not modulate neuronal activity in the normally functioning nigro-striatal system. However, these findings do not rule out the possibility that the pathway may exert an influence on SN DA neurones which project to the mesolimbic and cortical limbic regions, as suggested recently (Fuxe, Hökfelt, Ljungdahl, Agnati, Johansson and Perez de la Mora, 1975). This group of researchers reported that DA turnover in these areas was inhibited by the systemic administration of the proposed GABA agonist β-(p-chlorophenyl)-gamma-aminobutyric acid and the GABA-T inhibitor, aminooxyacetic acid. Moreover, these drugs were also found to block the increase in DA turnover induced by pimozide, a potent DA receptor blocker. The normal and pimozide-induced increase in the striatum were both unaffected by the administration of these drugs. Of course it is possible that these GABA effects were due to a direct action of these drugs on the terminals of the limbic DA system.

The effect of haloperidol on striatal DA metabolism is well documented. Both dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the acidic metabolites of DA, are considerably elevated following the administration of this neuroleptic (Andén, Roos and Werdenius, 1964; Sharman, 1966; Roth, Murrin and Walters, 1976). The rise in striatal DOPAC, found in the present study following 1 mg./kg. (300%) was similar to that reported recently (Roth, Murrin and Walters, 1976). These workers also concluded that this metabolite was the better index of the impulse flow in the dopaminergic neurones.

In the present study haloperidol was found to cause a marked increase in the striatal concentration of both these metabolites in unlesioned animals and animals lesioned in the striato-nigral pathway. This increase in metabolite concentrations was completely unaltered by prior lesioning of the ipsilateral striato-nigral pathway. These findings are in agreement with a previous study which claimed that haloperidol-induced increases in forebrain HVA levels were not abolished by prior large-scale lesioning of the crus cerebri. These lesions were reported not to damage the axons of the nigro-striatal system since SN cells on the lesioned side did not show signs of retrograde degeneration (Bedard and Larochelle, 1973). It is impossible to compare these lesions with the ones reported here since these workers presented their histological data from horizontal sections. But in all likelihood the extensive nature of the former lesions probably ensured that fibres of the striato-nigral pathway were interrupted. Thus, it is unlikely that the facilitatory effect of haloperidol on striatal DA metabolism is mediated via the striato-nigral pathway following post-synaptic DA receptor blockade in the striatum. It would be interesting to continue these experiments and investigate whether in fact haloperidol still increases the firing rate of SN DA neurones in these striato-nigral lesioned animals. Although haloperidol, in large doses (10 mg./kg.), has been reported to decrease SN GABA concentrations by 19% (Kim and Hassler, 1975) it is known that much smaller doses of haloperidol are able to increase DA cell firing rate to a maximum (Bunney et al., 1973 (a,b)). Therefore, if the striato-nigral pathway were responsible for the "feedback" control of the DA neurones in the SN then one would expect these GABA changes to occur at much lower doses of haloperidol.

Although recent evidence has suggested that neuroleptics may act directly on DA neurones, either on the cell bodies themselves (Bunney and Aghajanian, 1973; Groves et al., 1975) or on the terminals located in the striatum (Kehr et al., 1972; Walters and Roth, 1974; Seeman and Lee, 1974, 1975) there still exists a certain difficulty in explaining a few findings solely by a presynaptic mode of action. For example, chlorpromazine is known to increase striatal ACh turnover in rats with almost complete chronic lesions placed in the ipsilateral nigro-striatal DA system (Guyenet et al., 1975). This finding does strongly suggest that a post-synaptic site of action, most likely on ACh neurones in the striatum, may be important. Another finding difficult to explain by a presynaptic mode of action is the blocking effect of antimuscarinic drugs on the neuroleptic-induced increase in striatal DA metabolism (O'Keefe, Sharman and Vogt, 1970; Andén and Bedard, 1971; Andén, 1972). As with the neuroleptic drugs, it is uncertain whether antimuscarinic drugs act predominantly on neurones in the striatum or SN. This evidence for an effect of neuroleptic drugs on striatal ACh does support a neuronal "feedback" mechanism for the mode of action of these drugs, involving a striatal cholinergic interneurone. Further studies are required to investigate the site of action of these anticholinergic drugs in the striatum.

In summary, the entire extent of the striato-nigral pathway in the rat has been mapped out autoradiographically using the ^{3}H-leucine neuroanatomical tracing technique. The fibres originating from cell bodies in both dorsal and ventral regions of the striatum were found

to course caudally in the ventro-medial regions of the internal capsule and crus cerebri before terminating topographically in the zona reticulata region of the ipsilateral substantia nigra.

The course of the striato-nigral pathway was lateral to that of the ipsilateral nigro-striatal system and it proved possible to lesion the former without causing damage to the dopaminergic system. Rats with chronic, unilateral lesions placed in the region of the striato-nigral pathway, at the level of the mammilary bodies, showed a marked turning behaviour, towards the lesioned side, when treated with relatively high doses of apomorphine (0.5-5.0 mg./kg.) or amphetamine (2 mg./kg.). These lesions also reduced GABA concentration in the ipsilateral SN by 40-50%, although there was a considerable variation in the estimation of this transmitter in the non-lesioned side of individual animals.

Biochemical studies on these lesioned rats showed that striato-nigral lesions did not significantly alter either normal or haloperidol-enhanced DA metabolism in the ipsilateral striatum.

CHAPTER VI

GENERAL DISCUSSION

GENERAL DISCUSSION

The intention of this final discussion is to present a more general view of the present experimental findings along with other more recently reported results from laboratories elsewhere and comment on their implications to both ongoing and future research into the functional importance of the SN DA systems in the brain. In recent years a vast literature has accumulated concerning various aspects of these neurones and one is struck by the sheer diversity of the biochemical and behavioural functions in which DA has been suggested to be important, including the control of motor behaviour (Hornykiewicz, 1966; Ungerstedt, 1971,b,c); intracranial self-stimulation (Crow, 1972); regulation of food and water intake (Zigmond and Stricker, 1972); the uptake of glucose into brain cells (Schwartz, Sharp, Gunn and Evarts, 1976); schizophrenia (Snyder, Banerjee, Yamamura and Greenberg, 1974); short term memory and learning (Zis, Fibiger and Phillips, 1973; Routtenberg and Holzman, 1973). The postulation of a unifying hypothesis which encompasses this myriad of functions has not been forthcoming even from the most distinguished workers in the field and if progress is to be made in this direction it is vitally important at the outset to distinguish the possible contribution of other brain systems to these diverse functions from that of a DA involvement.

With the recent emergence of the neurotoxin 6-OH DA (Ungerstedt 1968, 1971) it became possible to perform relatively specific and discrete lesions of the nigral DA neurones and fibres, (Hökfelt and Ungerstedt, 1973); and whilst many neurobiologists have adopted this

chemical lesioning technique in addition to the traditional electrolytic and electrocoagulative procedures, it is apparent to the present author that experiments in which the SN is either lesioned or stimulated, as currently reported in the literature, are open to a number of criticisms. In the first instance, it is clear that little emphasis is placed on the extent of these lesions in terms of the resulting DA depletion in the various terminal areas in the extrapyramidal, mesolimbic and cortical areas (Ungerstedt, 1971(a); Hökfelt, Ljungdahl, Fuxe and Johansson, 1973; Berger, Thierry, Tassin and Moyne, 1976). By way of an example, it has recently been reported that rats with a DA depletion of over 95% in both the corpus striatum and telencephalon, following intraventricular injection of 6-OH DA, show an initial phase of adipsic and aphagic behaviour followed by a gradual recovery of almost normal ingestive behaviour but with an underlying deficit in both food and water intake, especially in stressful situations (Stricker and Zigmond, 1974). These workers suggested that the residual brain DA systems (2-5% of control) can largely compensate for the large loss of DA and thereby almost restore normal function. In their work, DA concentration was estimated in only two brain areas, namely the corpus striatum and the telencephalon, and it is apparent that their results provide very scant evidence as to which particular DA projection may be involved in this severe syndrome. It may be that DA depletion in a discrete DA projection, eg. the frontal cortex, is a crucial factor in the onset of these behavioural deficits, since it has been reported that lesions placed there result in a very similar syndrome (Kolb and Nonneman, 1975). Similar criticisms, in most cases can be levelled

at experiments in which the SN DA neurones are lesioned either chemically or electrolytically. In all likelihood the behavioural changes observed following either lesioning or stimulation in the SN will be closely related to the particular DA projection system most affected. Therefore, it is going to become increasingly important for researchers to present some kind of detailed biochemical profile of brain DA concentration following these kind of experimental procedures, in a bid to best assess which biochemical and behavioural changes are associated with a given DA projection system.

A second problem facing research into the functional importance of nigral DA neurones is the ever-increasing number of reported pathways coursing through the zona compacta region; as if this area was a focal point through which a large number of both ascending and descending pathways pass. The descending pathway, with cell bodies of origin in the lateral hypothalamus is a particular case in point (Fig.36). Recently, other pathways, originating in the frontal cortex (Clavier and Corcoran, 1976) and the dorsal tegmental region (Clavier and Routtenberg, 1976(a)) have been reported to course through the SN. These researchers have proposed, on reasonably sound anatomical evidence, that these pathways are involved in intracranial self-stimulation behaviour (ICSS) and have queried the importance of DA neurones in this behaviour. Thus, one does not know whether it is stimulation of these non-dopaminergic pathways, dopaminergic pathways or both that is of vital importance in supporting nigral ICSS. 6-OH DA-induced lesioning of the DA cell bodies may not help in clarifying this problem since the currently used intracerebral pressure

injections of this compound which usually deliver 2ul over a period of 2 minutes (Ungerstedt, 1971), may cause a substantial amount of non-specific damage to fibres of passage. Few workers report on the possible non-specific damage resulting from such injection procedures but in future it will be essential to further investigate this kind of problem if progress is to be made in the understanding of which pathways are important in ICSS behaviour.

DA neurones and intracranial self-stimulation behaviour

Since Crow (1973) suggested that both dopaminergic and noradrenergic pathways may be important in ICSS behaviour, many researchers have been prompted to further investigate this possibility. Crow's original suggestion that the NA-containing cell bodies in the region of the locus coeruleus supported ICSS behaviour has been questioned recently by a number of workers. In particular, ICSS behaviour supported by electrodes in the vicinity of the locus coeruleus is not attenuated by 6-OH DA-induced lesions of the dorsal noradrenergic pathway, arising from this nucleus (Clavier, Fibiger and Phillips, 1976). As further supportive evidence of the non-involvement of NA-containing pathways in brain stem ICSS behaviour it has also been reported that self stimulation through electrodes situated in the region of the dorsal noradrenergic pathway is not affected by lesioning of the ipsilateral locus coeruleus or the ventral noradrenergic bundle (Clavier and Routtenberg, 1976 (b)). Thus, these recent experiments have placed a large question mark against the importance of noradrenergic pathways in brain stem ICSS behaviour. It has been reported that ICSS behaviour from electrode

sites in the dorsal tegmental area is attenuated by lesions placed in the medial forebrain bundle (Clavier and Routtenberg, 1976(b)) and although either ascending or descending pathways could be involved it is interesting to note that efferent fibres of the lateral hypothalamic neurones course through this dorsal tegmental region (Fig.36). It is a consistent finding that self-stimulation rates from dorsal tegmental sites are much lower than those obtained from sites in the lateral hypothalamus and SN (M.Mitchell, personal communication) and this is consistent with a stimulation of lateral hypothalamic efferent fibres at more caudal sites, where the fibres are more scattered and fewer in number, and which one would intuitively predict would support lesser bar-pressing rates.

The involvement of DA-containing pathways in ICSS behaviour is well supported by anatomical evidence in that there is a reasonably good correlation between positive electrode sites in the central regions of the midbrain and diencephalon and the location of DA-containing neurones (Crow, 1972; Prado-Alcala, Kent and Reid, 1975). Early pharmacological studies, using drugs which either inhibited or enhanced catecholamine pathway activity (see Crow, 1973) were instrumental in tipping the balance in favour of an involvement of these pathways in ICSS behaviour. However, a more recent and refined drug study, using the suggested specific DA receptor antagonist pimozide (Anden et al., 1970), has demonstrated that ICSS behaviour from both presumed DA sites (nucleus accumbens) and NA sites (dorsal tegmental bundle) is markedly depressed by pretreatment with this drug, (Phillips, Brooke and Fibiger, 1975). This finding highlights

the major difficulty of distinguishing between a specific blockade by a drug of a possible reward system and a general disruption of the motor sequences required to make operant responses. Although the bulk of the pharmacological data in support of the involvement of DA pathways in ICSS behaviour is open to this form of criticism it is important to emphasise that pimozide treatment does not always necessarily attenuate operant responding (Wise, 1976). He reported that rats which had been trained to self-administer amphetamine intravenously by pressing a bar, increased their bar-pressing rates following the administration of pimozide. Just how amphetamine self-administration relates to ICSS behaviour is uncertain but for the present this evidence does suggest that this form of "drug reward" may be due to amphetamine acting on central dopaminergic pathways.

In general terms, lesion studies in the same mould as those described in the studies of the involvement of NA-containing pathways in ICSS behaviour are technically more difficult to perform in the SN, due to the many other fibre systems coursing through this region, including serotoninergic and noradrenergic pathways (Ungerstedt, 1971(a)). One recently adopted approach is to implant electrodes in a known DA terminal region, eg. the corpus striatum, demonstrate that ICSS behaviour is supported and subsequently lesion the cell bodies of the nigro-striatal system (Phillips, Carter and Fibiger, 1976). Before commenting on the results of this experiment it is instructive to delineate the marked differences in the nature of the ICSS behaviour supported by electrodes implanted in the striatum and the SN. Whilst SN-implanted animals are characterised by ease of training and a

general avidity in operant responding, the former display generally much slower response rates after a prolonged training period (mean, 8 days). Whether these differences are due either to the involvement of different neuronal systems or the "intensity" of the reward elicited by stimulation of the same system i.e. the nigro-striatal system, is at the moment uncertain. 6-OH DA-induced destruction of the ipsilateral DA neurones in these animals showing striatal ICSS behaviour chronically depressed (by 80%) the bar-pressing rates of these animals. Destruction of the contralateral SN DA system initially depressed the response rates by the same amount as the ipsilateral lesions but the rats gradually increased the rate of operant responding, over a period of 20 days, to 72% of the pre-lesion value. Although unilateral DA denervation did significantly reduce brain stimulation reward it does appear that these lesions also produced an initial general disruption of bar-pressing behaviour. However, the chronic attenuation of operant responding caused by the ipsilateral lesion does support the involvement of the nigro-striatal system in striatal ICSS behaviour. This kind of experimental approach is, of course, open to the criticisms previously mentioned concerning the interpretation of results following intra-nigral injections of 6-OH DA. Perhaps a better approach, although far from ideal, would be to study ICSS behaviour from electrodes sited in the SN, both before and after lesions placed in the different DA terminal regions. This would facilitate separation of the effects of lesions on non-dopaminergic pathways currently, thought to be important in ICSS behaviour, from those resulting after destruction of specific DA terminal regions. At present the evidence for an involvement of DA

pathways in ICSS behaviour is more concrete than that available for NA-containing pathways. It is to be hoped that further investigations will bolster this evidence.

In spite of the doubts surrounding the involvement of brain catecholamine-containing pathways in ICSS behaviour it is still surprising to discover that so few investigators have tested Crow's original hypothesis electrophysiologically. Apart from this present study, only one other group has studied the possibility of olfactory stimuli influencing DA neurones (Mogenson and Faiers, 1976.). In contrast to the present study (Chapter III), these workers reported, in urethane-anaesthetised rats, that A10 nigral neurones were predominantly excited by olfactory bulb stimulation (mean latency, 23±6 msec.; range, 5-80 msec.). No units in either the zona compacta or the zona reticulata were affected. However, they did not investigate the possibility of a non-specific stimulation of the A10 neurones. As mentioned in the Discussion of Chapter III, the problem with these kind of experiments is in distinguishing between neuronal responses to specific sensory inputs and non-specific arousal. Crow 1973 suggested that nigral DA neurones were influenced by incentive stimuli i.e. bearing the promise of a reward, and it is obvious that a further test of his elegant hypothesis will require more subtle electrophysiological studies performed on conscious animals, of the same design as those pioneered by Phillips and Olds, 1969. They showed that midbrain units could make discriminating responses according to the significance of different sensory signals. Although such experiments are sufficient to daunt even the most enthusiastic

and skilful of research workers it is apparent that this is the kind of gap that must be bridged if any inroads are to made into physiological psychology. In addition to performing studies on the influence of incentive stimuli on DA neuronal activity it may also be important to study the effect of deficit signals (eg. thirst and hunger) on these neurones since they have implicated in the regulation of food and water intake (Stricker and Zigmond, 1974).

DA neurones and the control of motor behaviour

The major DA projection system terminates in the corpus striatum, a nucleus thought to be important in the control of motor behaviour (Kemp and Powell, 1971), especially in the initiation and control of movement (Mogenson and Phillips, 1976). It is possible, indeed highly likely that the nigro-striatal system has a vital role in the control of the output of the corpus striatum. The major output of this nucleus, as shown in Chapter V, is the striato-nigral pathway and from the biochemical and anatomical data obtained it does seem unlikely that it exerts a tonic inhibitory influence on DA neurones in the SN. Instead the author favours the possibility that the terminal area of the striato-nigral pathway, the zona reticulata region, acts as a synaptic relay between the striatum and the ventrolateral nucleus of the thalamus. This is supported by electrophysiological evidence which has demonstrated the existence of a functional striato-nigral-thalamo pathway (Deniau, Feger and Le Guyader, 1976). Thus, the nigro-striatal pathway may be important in influencing some aspects of motor behaviour via this functional pathway eg. stereotyped behaviour (Ernst, 1967). It is known that unilateral 6-OH DA-induced

lesions in the SN do not cause chronic overt motor disturbances in the rat unless the animal is treated with a drug which influences the postulated supersensitive striatal DA receptors on the lesioned side (Ungerstedt, 1971(c)). This drug treatment results in a characteristic turning behaviour, away from the side of the lesion. It is still unclear as to why there are so few signs of motor imbalance in the untreated lesioned rat. Even although the rat's open field behaviour is at first glance normal it is obvious that there is a severe sensorimotor deficit present which only becomes apparent when the animal is presented, on the contralateral side, with stimuli which usually elicit an orientating response to the stimulus. Whether such deficits are primarily sensory or motor is still uncertain but it is interesting to mention that this same kind of "sensory neglect" has been observed in rats with unilateral lesions placed in the striato-nigral pathway (Tulloch, unpublished observation). This does support the possibility that the primary deficit is on the motor side. In the future it will be interesting to investigate electrophysiologically possible neuronal abnormalities which may underlie this syndrome. Lesioning of the nigro-striatal system causes an increase in cell firing rate in the ipsilateral striatum (Arbuthnott, 1974) and it is reasonable to assume that this increase in activity may be reflected in a change in impulse traffic in the striato-nigral pathway, if one accepts that there is functional connection between the dopaminergic input and the gabaminergic output of the striatum. Possible changes in striatal output could perhaps be studied electrophysiologically by recording single neurones in the zona reticulata on both the lesioned and intact sides. It is even

possible that similar studies would provide evidence for DA receptor sensitivity which has been postulated to occur following nigral DA lesions (Ungerstedt, 1971(c)) by recording the activity of zona reticulata cells and studying their responsiveness, if any, to intra-striatal injections of a DA agonist drug.

Self-regulation of DA neurones

The possibility that the dendrites of nigral DA neurones release DA is supported anatomically (Björklund and Lindvall, 1975) and biochemically (Geffen, Jessel, Cuello and Iversen, 1976; Korf, Zielman and Westerink, 1976) but the functional importance of this release is at present unknown, Groves et al., 1975, have suggested that DA neurones self-inhibit each other via this dendritic release of DA. This presumably means that these neurones may exert a quite considerable influence over their own firing rate as well as that of their neighbours. The further elucidation of these tentative interconnections within the SN will demand the application of micro-stimulation and electrophysiological techniques. At the moment, if one accepts that the DA cells do not fire in a synchronous manner when influenced by afferent inputs and if one acknowledges the possibility that certain DA cells may be active whilst others are actively suppressed, then the importance of an intra-nigral control mechanism, as outlined above, becomes apparent. However, no evidence is available to support this tentative suggestion. From the limited number of electrophysiological studies on nigral DA neurones two general findings emerge. Firstly the majority of cells appear to be spontaneously active with a firing frequency confined to a relatively

narrow bandwidth of approximately 1-14 Hz., depending on the anaesthetic used. Secondly, neurones in the different areas of the zona compacta and VTA all appear to have the same firing characteristics with no apparent clustering of cells having the same firing rate. It may be very important to the animal to have a similar optimum activity in the various DA projections in order to maintain normal behaviour. This is supported by the development of apparent compensatory mechanisms following either hypo-function or hyper-function of DA systems eg. the development of supersensitivity in striatal DA receptors following lesioning of the nigro-striatal pathway and the inhibition of DA cell firing rate by DA receptor stimulation (Chapter II). The importance of an optimum level of DA cell activity in the normal behaviour of the animal is unknown. Perhaps it may maintain a critical level of arousal in order that the animal can perform "get up and go" tasks when confronted with incentive or stressful stimuli.

The present HRP study provided evidence of hitherto unsuspected nigral afferent inputs. Although this kind of approach is remarkably tedious it is becoming obvious that neuroanatomical studies are required in the mapping out of the neural inputs to the SN. The majority of injection sites in the present HRP study were well localised to the A9 region but in the future it will be necessary to continue these studies and compare the described HRP-labelled cell distribution with that observed following injection of HRP into the A10 and A8 areas. This kind of study would provide evidence as to whether the neural projection to the various nigral DA-containing cell

areas were similar or not. In view of the diverse projections of the nigral DA cell it is unlikely that all the cells are similarly influenced by a given neuronal input although it is likely that all the DA cells influence neurones in their terminals areas in the same manner ie. an inhibitory effect (see Krynevic, 1975). Therefore, in the future it will be important to establish the neuronal pathways projecting to different areas of the SN and investigate how these inputs affect DA cell activity in the various DA terminal areas as well as the behaviour of the animals.

BIBLIOGRAPHY

Aghajanian, G.K., and Bunney, B.S. (1973).
in "Frontiers of Catecholamine Research", pp.643-648. Eds., Usdin, E., and Snyder, S.H. Pergamon Press.

Akagi, K., and Powell, E.W. (1968)
J.Comp.Neurol., 132, 263-274

Alderman, J.L., and Schellenberger, M.K. (1974)
J.Neurochem., 22, 937-940

Amassian, V.E., and De Vito, R. (1954).
J.Neurophysiol., 17, 575-603

Anand, B.K., and Brobeck, J.R. (1951).
Yale J. Biol.Med., 24, 123-140

Andén, N.-E. (1972)
J.Pharm.Pharmacol., 24, 905-906

Andén, N.-E. (1974)
Naunyn-Schmiedeberg's Arch.exp.Path.Pharmak., 283, 417-424

Andén, N.-E., and Bedard, P. (1971)
J.Pharm.Pharmac., 23, 460-462

Andén, N.-E., Bedard, P., Fuxe, K., and Ungerstedt, U. (1972)
Experienta, (Basel), 28, 300-302

Andén, N.-E., Butcher, S.G., Corrodi, H., Fuxe, K., and Ungerstedt, U. (1970)
Europ.J.Pharmacol., 11, 303-314

Andén, N.-E., Carlsson, A., Dahlström, A., Fuxe, K., Hillarp, N.-Å., and Larsson, K. (1964).
Life Sci., 3, 523-530

Andén, N.-E., Carlsson, A., and Häggendahl, (1969)
Ann.Rev.Pharm., 9, 119-134

Andén, N.-E., Corrodi, H., Fuxe, K., and Ungerstedt, U. (1971).
Europ.J.Pharmacol., 15, 193-199

Andén, N.-E., Dahlström, A., Fuxe, K., and Larsson, K. (1965)
Life Sci., 4, 1275-1279

Andén, N.-E., Dahlström, A., Fuxe, K., and Larsson, K. (1966)
Acta.pharmacol.(KbH), 24, 263-274

Andén, N.-E., Roos, B.E., and Werdenius, B. (1964)
Life Sci., 3, 149-158

Andén, N.-E., Rubenson, A., Fuxe, K., and Hökfelt, T. (1967)
J.Pharm.Pharmac., 21, 263-264

Andén, N.-E., and Stock, G. (1973).
Naunyn-Schmiedeberg's Arch.exp.Path.Pharmak., 279, 89-92

Arbuthnott, G.W. (1974)
J.Physiol.(Lond.), 239, 121-122

Arduinni, A., and Moruzzi, G. (1953)
Electroenceph.clin.Neurophysiol., 5, 243-250

Axelrod, J., and Tomchick, R. (1958)
J.Biol.Chem., 233, 702-705

Balcom, G.J., Lennox, R.H., and Meyerhoff, J.L. (1975)
J.Neurochem., 24, 609-613

Barraclough, C.A., and Cross, B.A. (1963)
J.Endocrin., 26, 339-359

Bedard, P., and Larochelle, L. (1973)
Exp.Neurol., 41, 314-322

Bell, C., Sierra, G., Buendia, N., and Segundo, J.P.
J.Neurophysiol., 27, 961-987

Ben-Ari, Y., and Kelly, J.S. (1976)
J.Physiol., 256, 1-22

Berger, B., Tassin, J.P., Blanc, G., Moyne, M.A., and Thierry, A.M. (1974)
Brain Res., 81, 332-337

Berger, B., Thierry, A.M., Tassin, J.P., and Moyne, M.A. (1976)
Brain Res., 106, 133-145

Berridge, T.L., and Sharman, D.F. (1974)
Brit.J.Pharmacol., 50, 156-158

Bertler, A., and Rosengren, E. (1959)
Acta physiol.scand., 47, 350-361

Besson, M.J., Cheramy, A., Feltz, P., and Glowinski, J. (1969)
Proc.Nat.Acad.Sci., 62, 741-748

Besson, M.J., Cheramy, A., Feltz, P., and Glowinski, J. (1971)
Brain Res., 32, 407-424

Bisset, G.W., Errington, M.L., and Richards, C.D. (1973)
Brit.J.Pharmacol., 48, 263-272

Björklund, A., and Lindvall, O. (1975)
Brain Res., 83, 531-537

Black, W.C., and Cooper, B.R. (1970)
Physiology and Behaviour, 5, 1405-1409

Bloom, F.E., Costa, E., and Salmoiraghi, G.C. (1965)
J.Pharmacol.Exp.Therap., 150, 244-252

Bunney, B.S., and Aghajanian, G.K. (1973)
in "Frontiers of Catecholamine Research", pp.957-962. Eds., Usdin, E., and Snyder, S.H. Pergamon Press.

Bunney, B.S., and Aghajanian, G.K. (1975)
In "Pre- and Postsynaptic Receptors", pp.89-121. Eds., Usdin, E., and Bunney, W.E. Marcel Dekker, New York.

Bunney, B.S., and Aghajanian, G.K. (1976)
Science, 192, 391-393

Bunney, B.S., Walters, J.R., Roth, R.H., and Aghajanian, G.K. (1973(a)).
J.Pharmacol.Exp.Therap., 185, 560-571

Bunney, B.S., Aghajanian, G.K., and Roth, R.H. (1973 (b)).
Nature New Biology, 245, 123-125

Broadwell, R.D. (1975)
J.Comp.Neurol., 164, 389-410

Broadwell, R.D., and Brightman, M. (1976)
J.Comp.Neurology, 166, 257-284.

Brown, P.B. (1973)
in "Electronics for Neurobiologists", p.402

Campbell, H.J. (1968)
Nature, 218, 104-105

Carlsson, A. (1975)
in "Pre- and Postsynaptic Receptors" pp.49-65. Eds., Usdin, E., and Bunney, W.E. Marcel Dekker. New York.

Carlsson, A., Falck, B., and Hillarp, N.-Å. (1962)
Acta physiol.scand., 56, Suppl.196

Carlsson, A., Falck, B., Fuxe, K., and Hillarp, N.-Å. (1964)
Acta physiol.scand., 60, 112-119

Carlsson, A., Fuxe, K., Hamberger, B., and Lindqvist, M. (1966)
Acta physiol.scand., 67, 481-497

Carlsson, A., Kehr, W., Lindqvist, M., Magnusson, T., and Atack, C.V. (1972).
Pharmacol.Rev., 24, 371-384

Carlsson, A., Lindqvist, M., Magnusson, T., and Waldeck, B. (1958)
Science, 127, 471

Carlsson, A., Lindqvist, M., and Magnusson, T. (1960)
in: "Ciba Found.Symp. on Adrenergic Mechanisms.", pp.432-439.
Eds.: Vane, J.R., Wolstenholme, G.W., and O'Connor, M.
J. and A. Churchill Ltd.

Carlsson, A., and Lindqvist, M. (1962)
Acta physiol.scand., 54, 87-94

Carlsson, A., and Lindqvist, M. (1963)
Acta pharmacol.toxicol., 20, 140-144

Clavier, R.M., and Corcoran, M.E. (1976)
Brain Res., 113, 59-69

Clavier, R.M., Fibiger, H.C., and Phillips, A.G. (1976)
Brain Res., 113, 71-81

Clavier, R.M., and Routtenberg, A. (1976(a)).
Brain Res., 105, 325-332

Clavier, R.M., and Routtenberg, A. (1976(b)).
Brain Res., 101, 251-271

Connor, J.D., (1968)
Science, 160, 899-900

Connor, J.D. (1970)
J.Physiol., 208, 691-703

Corrodi, H., Fuxe, K., and Hökfelt, T. (1967)
Life Sci., 6, 767-774

Cowan, W.M., Gottlibe, D.I., Hendrickson, A.E., Price, J.L., and
Woolsey, T.A.
Brain Res., 37, 21-51

Cragg, B.G. (1961)
Exp.Neurol., 3, 388-409

Crossman, A.R., Walker, R.J., and Woodruff, G.N. (1973)
Brit.J.Pharmac., 49, 696-698

Crow, T.J. (1969)
Nature, 224, 709-710

Crow, T.J. (1971)
Experientia (Basel), 27, 622

Crow, T.J. (1972)
Brain Res., 36, 265-273

Crow, T.J. (1973)
Psychological Med., 3, 66-73

Crow, T.J., and Arbuthnott, G.W. (1972)
Nature New Biology, 238, 245-246

Dahlström, A., and Fuxe, K. (1964(a)).
Acta physiol.scand., 62, Suppl.1-55.

Dahlström, A., and Fuxe, K. (1964(b)).
Experientia, (Basel)., 20, 398-399

Dahlström, A., and Fuxe, K. (1964 (c)).
Acta physiol.scand., 60, 293-295

Dahlström, A., and Fuxe, K. (1965)
Acta physiol.scand., 64, Suppl. 247, 39-85.

Da Prada, M., and Pletscher, A. (1966)
Experientia, (Basel), 22, 1-5

Deniau, J.M., Feger, J., and Le Guyader, C. (1976)
Brain Res., 104, 152-156

De Vito, J.L., Clausing, K.W., and Smith, O.A. (1974)
Brain Res., 82, 269-271

Dray, A., Oakley, N.R., and Simmonds, M.A. (1975(a)).
J.Pharm.Pharmac., 27, 627-629

Dray, A., Fowler, L.J., Oakley, N.R., Simmonds, M.A., and Tanner, T. (1975(b)).
Brit.J.Pharmac., 55, 288p.

Dray, A., and Gonye, T.J. (1975)
J.Physiol., 246, 88-89p.

Dray, A., Gonye, T.J., and Oakley, N. (1976)
J.Physiol., 259, 825-849

Dray, A., Gonye, T.J., Oakley, N.R., and Tanner, T. (1976)
Brain Res., 113, 45-57

Dresse, A. (1966)
Life Sci., 5, 1003-1014

Eranko, O., (1955)
Acta endocrin., 18, 180-188

Ernst, A.M. (1965)
Psychopharmacoligia, 7, 391-399

Ernst, A.M., and Smelik, P.G. (1966)
Experientia, 22, 837-838

Ernst, A.M. (1967)
Psychopharmacologia (Berl.), 10, 316-323

Euler, U.S. von. (1947)
Acta physiol.scand., 12, 73-97

Falck, B. (1962).
Acta physiol.scand., 56, Suppl. 197

Falck, B., Hillarp, N.A., Thieme, G., and Torp, A. (1962)
J.Histochem.Cytochem., 10, 348-354

Falck, B., and Owman, C. (1965)
Acta Univ.lund., II, No.7, 5-23

Fahn, S., and Cote, L.J. (1968)
J.Neurochem., 15, 209-213

Farnebo, L.-O., and Hamberger, B. (1970)
J.Pharm.Pharmacol., 22, 855-857

Farnebo, L.-O., and Hamberger, B. (1971)
Acta physiol.scand., Suppl.371, 35-44.

Faull, R.L.M., and Carmen, J.B. (1968)
J.Comp.Neurol., 132, 73-92

Feger, J., and Ohye, C. (1975)
Brain Res., 89, 155-159

Feltz, P. (1971)
Canadian J. of Physiol.Pharmacol., 49, 1113-1115

Feltz, P., and McKenzie, J.S. (1969)
Brain Res., 13, 612-616

Feltz, P., and De Champlain, J. (1972)
Brain Res., 43, 595-600

Ferrer, N.G. (1969)
J.Comp.Neurol., 137, 309-326

Fibiger, H.C., Zis, A.P., and McGeer, E.G. (1973)
Brain Res., 55, 135-148

Finch, L., and Haeusler, G. (1973)
Europ.J.Pharmacol., 21, 264-270

Fink, R.P., and Heimer, L. (1967)
Brain Res., 4, 369-374

Fonnum, F., Grofova, I., Rinvik, E., Storm-Mathisen, J., and Walberg, F., (1974)
Brain Res., 71, 77-92

Foote, W.E., Sheard, M.H., and Aghajanian, G.K. (1969)
Nature, 222, 567-569

Freund-Mercier, M.J., Richard, P., and Miro, J.L. (1975)
Brain Res., 89, 149-154

Frigyesi, T.L., and Purpura, D.P. (1967)
Brain Res., 6, 440-456

Frigyesi, T.L., and Szabo, J. (1975)
Exp.Neurol., 49, 123-139

Fuxe, K. (1965)
Z.Zellforsch., 65, 573-596

Fuxe, K., Hökfelt, T., Johansson, O., Jonsson, G., Lidbrink, P., and Ljungdahl, A. (1974)
Brain Res., 82, 349-355

Fuxe, K., Hökfelt, T., Ljungdahl, Å., Agnati, L., Johansson, O., and Perez de la Mora, M. (1975)
Medical Biology, 53, 177-183

Geffen, L.B., Jessel, T.M., Cuello, A.C., and Iversen, L.L. (1976)
Nature, 260, 258-260

Gibson, S., McGeer, E.G., and McGeer, P.L. (1970)
Exp.Neurol., 27, 283-290

Glowinski, J., Agid, Y., and Javoy, F. (1973)
Nature New Biology, 245, 150-152

Goldstein, M., Anagnoste, B., and Owen, W.S. (1966)
Life Sci., 5, 2171-2176

Goswell, M.J., and Sedgwick, E.M. (1971)
J.Physiol. (Lond.), 218, 84-85

Graham, R.C., and Karnovsky, M.J. (1966)
J.Histochem.Cytochem., 14, 291-302

Green, J.D. (1958)
Nature, 182, 962-963

Grofová, I. (1975).
Brain Res., 91, 286-291

Grofová, I., and Rinvik, E. (1970)
Exp.Brain Res., 11, 249-262

Groves, P.M., Wilson, C.J., Young, S.J., and Rebec, G.V. (1975)
Science, 190, 552-529

Gudden, B. (1870)
Arch.Psychiat.Nervenkr., 2, 693-723

Guillery, R.W. (1957)
J.Anat.(Lond.), 91, 91-115

Guyenet, P.G., Agid, Y., Javoy, F., Beaujouan, J.C., Rossier, J., and Glowinski, J. (1975)
Brain Res., 84, 227-244

Hadju, F., Hassler, R., and Bak, I.J. (1973)
Z.Zellforsch., 146, 207-221

Harris, J.E., Baldessarini, R.J., and Morgenroth, V.H. (1974)
Nature, 252, 156-158

Hattori, T., McGeer, P.L., Fibiger, H.C., and McGeer, E.G. (1973)
Brain Res., 54, 103-114

Hattori, T., Fibiger, H.C., and McGeer, P.L. (1975)
J.Comp.Neurol., 162, 487-504

Heimer, L. (1972)
Brain, Behav.Evol., 6, 484-523

Herrick, C.J. (1948)
in "The brain of the Tiger Salamander, Ambystoma Tigrinum".
University of Chicago Press

Hjorth-Simonsen, A. (1970)
Stain Technology, 45, 199-204

Hökfelt, T., Ljungdahl, Å., Fuxe, K., and Johansson, O. (1973)
Science, 184, 177-179

Hökfelt, T., and Ungerstedt, U. (1973)
Brain Res., 60, 269-297

Holtz, P. (1950)
Acta physiol.scand., 20, 354-362

Horn, A.S., Synder, S.H. (1971)
Mol.Pharmacol., 7, 66-80

Hornykiewicz, O. (1966)
Pharm.Rev., 18, 925-964

Huang, Y.H., and Routtenberg, A. (1971)
Physiology and Behaviour, 7, 419-432

Hübel, D.H. (1957)
Science, 125, 549-550

Hull, C.D., Barnardi, G., and Buchwald, N.A. (1970)
Brain Res., 22, 163-179

Jones, E.G., (1975).
Brain Res., 85, 249-253

Jones, E.G. and Leavitt, R.Y. (1974)
J.Comp.Neurol., 154, 349-378

Jacobwitz, D.M., and Palkovits, M. (1974)
J.Comp.Neurol., 157, 13-28

Kanazawa, I., Miyota, Tokokura, Y., and Otsuka, M. (1973)
Brain Res., 51, 363-365

Kataoka, K., Bak, I.J., Hassler, R., Kim, J.S., and Wagner, A. (1974)
Exp.Brain Res., 19, 217-227

Kebabian, J.W., Petzgold, G.L., and Greengard, P. (1972)
Proc.Nat.Acad.Sci., 69, 2145-2149

Kehr, W., Carlsson, A., Lindqvist, M., Magnusson, T., and Atack, C.V. (1072)
J.Pharm.Pharmacol., 24, 744-747

Keller, H.H., Batholini, G., and Pletscher, A. (1973)
Europ.J.Pharmacol., 23, 183-186

Kemp, J.M. (1970)
Brain Res., 17, 125-128

Kemp, J.M., and Powell, T.P.S. (1971)
Philosophical Transactions of the Royal Society of London,
Series B262, 441-457

Kerkut, G.A., Shapira, A., and Walker, R.J. (1967)
Comp.Biochem.Physiol., 23, 729-748

Kim, J.S., Bak, I.J., Hassler, R., and Okada, Y. (1971)
Exp.Brain Res., 14, 95-104

Kim, J.S., and Hassler, R. (1975)
Brain Res., 88, 150-153

Klüver, H., and Barrera, E. (1953)
J.Neuropath.exp,Neurol., 12, 400-403

Kolb, B., Nonneman, A.J. (1975)
J.Comp.Physiol.Psychol., 88, 806-815

König, J.F.R., and Klippel, R.A. (1963)
in "The Rat Brain". Williams and Wilkins Co., Baltimore.

Komisaruk, B.R., and Beyer, C. (1972)
Brain Res., 36, 153-170

Korf, J., Aghajanian, G.K., and Roth, R.H. (1973)
Europ.J.Pharmacol., 21, 305-310

Korf, J., Zielman, M., and Westerink, B.H.C. (1976)
Nature, 260, 257-258

Kristensson, K., and Olsson, Y. (1971)
Brain Res., 29, 363-365

Kristensson, K., Olsson, Y., and Sjöstrand, J. (1971)
Brain Res., 32, 399-406

Krnjevic, K. (1975)
Advances in Neurology, 9, , Eds., Calne, D.B., Chase, T.N., and Barbeau, A. Raven Press, New York.

Kuypers, H.G., Kievit, J., and Groen-Klevant, A.C. (1974)
Brain Res., 67, 211-218

Lake, N. (1972)
Canadian Physiol., 3, 22-31

Lake, N. (1973)
Exp.Neurol., 41, 113-132

LaVail, J.H., (1975(a))
Federation Proceedings., 34, 1618-1624

LaVail, J.H. (1975(b))
in: "The Use of Axonal Transport for Studies of Neuronal Connectivity." pp.217-248. Eds: Cowan, M.D., and Cuenod, M. Elsevier Scientific Publishing Company.

LaVail, J.H., and LaVail, M.M. (1972)
Science. 176, 1416-1417

LaVail, M.M., and LaVail, J.H. (1975)
Brain Res., 85, 273-280

LaVail, J.H., Winston, K.R., and Tish, A. (1973)
Brain Res., 58, 470-477

Lenn, N. (1976)
J.Comp.Neurol., 166, 73-100

Levitt, M., Spector, S., Sjoerosma, A., and Udenfriend, S. (1965)
J.Pharm.exp.Therap., 148, 1-8

Lindvall, O., and Björklund, A. (1974)
Acta physiol.scand., Suppl. 412, 1-48

Ljungdahl, A., Hökfelt, T., Goldstein, M., and Park, D. (1975)
Brain Res., 84, 313-319

Machne, X., Calma, I., and Magoun, H.W. (1955)
J.Neurophysiol., 18, 547-558

MacLeod, P., (1972)
Handbook of Sensory Physiology, Vol.IV/1, pp.182-204. Elsevier.

Marshall, J.F., Turner, B.M., and Teitelbaum, P. (1971)
Science, 174, 523-525

Marshall, F.J., and Teitelbaum, P. (1974)
J.Comp.Physiol.Psychol., 86, 375-395

Marshall, J.F., Teitelbaum, P., and Richardson, J.S. (1974)
J.Comp.Physiol.Psychol., 87, 808-830

McGeer, E.G., Fibiger, H.C., Maler, L., Hattori, T., and McGeer, E.G. (1974)
Advances in Neurology, 5, 153-160. Eds. Calne, D.B., Chase, T.N., and Barbeau, A. Raven Press. New York.

McGeer, P.L., Grewaal, D.S., and McGeer, E.G. (1974)
Brain Res., 80, 211-217

McGeer, E.G., McGeer, P.L., Grewaal, D.S., Singh, V.K. (1975)
J.Pharmacol., 2, 143-152

McLennan, H. (1964)
J.Physiol., 174, 152-161

McLennan, H., and York, D.H. (1967)
J.Physiol., 189, 393-402

McNair, J.L., Sutin, J., and Tsubokawa, T. (1972)
Exp.Neurol., 37, 395-411

Millhouse, O. (1969)
Brain Res., 15, 341-363

Mizuno, N., and Nakamura, Y. (1974)
Brain Res., 65, 165-169

Mogenson, G.J., and Faiers, A.A. (1976)
in "Brain-Stimulation Reward." pp.579-585. Eds.: Wayquier, A., and Rolls, E.T. Elsevier.

Mogenson, G.J., and Phillips, A.G. (1976)
in "Progress in psychobiology and physiological psychology." Vol.6, pp.189-243. Ed., Sprague, J.M. Academic Press.

Mok, A.C.S., and Mogenson, G.J. (1974)
Brain Res., 77, 417-429

Montagu, K.A. (1957)
Nature, 180, 244-245

Morgenroth, V.H., Boadle-Biber, M., and Roth, R.H. (1974)
Proc.Nat.Acad.Sci., 71, 4283-4287

Motokizawa, F. (1973)
Electroenceph.clin.Neurophysiol., 35, 83-91

Motokizawa, F. (1974)
Exp.Neurol., 44, 135-144

Murrin, L.C., Morgenroth, V.H., and Roth, R.H. (1974)
The Pharmacologist, 16, 128

Murrin, L.C., and Roth, R.H. (1973)
The Pharmacologist, 15, 513

Nauta, W.J.H. (1958)
Brain, 81, 319-340

Nauta, W.J.H., and Gygax, P.A. (1953)
Stain Technology, 29, 91-93

Nauta, W.J.H., and Mehler, W.R. (1966)
Brain Res., 1, 3-42

Nauta, H.J.W., Kaiserman-Abramof, and Lasek, R.J. (1975)
Brain Res., 85, 373-384

Nauta, H.J.W., Pritz, M.B., and Lasek, R.J. (1974)
Brain Res., 67, 219-238

Nissl, F. (1892)
Allg.Z.Psychiat., 48, 197-198

Nybäck, H., and Sedvall, G. (1968)
J.Pharmacol.Exp.Therap., 162, 294-308

Nybäck, H., and Sedvall, G. (1971)
J.Pharm.Pharmac., 23, 322-326

O'Keefe, R., Sharman, D.F., and Vogt, M. (1970)
Brit.J.Pharmac., 38, 287-304

Palkovits, M., Brownstein, M., Saavedra, J.M., and Axelrod, J. (1974)
Brain Res., 77, 137-149

Papez, J.W. (1938)
J.Comp.Neurol., 69, 329-349

Pearson, J.D.M., and Sharman, D.F. (1975(a)).
Brit.J.Pharmac., 53, 143-148

Pearson, J.D.M., and Sharman, D.F. (1975(b)).
J.Neurochem., 24, 1225-1228

Pfaff, D.W. and Pfaffmann, C. (1969)
Brain Res., 15, 137-156

Phillips, A.G., Brooke, S.M., and Fibiger, H.C. (1975)
Brain Res., 85, 13-22

Phillips, A.G., Carter, D.A., and Fibiger, H.C. (1976)
Brain Res., 104, 221-232

Phillips, A.G., and Mogenson, G.J. (1969)
Physiology and Behaviour, 4, 195-197

Phillips, M.I., and Olos, J. (1969)
Science, 165, 1269-1271

Pickel, V.M., Segal, M., and Bloom, F.E. (1974)
J.Comp.Neurol., 155, 15-42

Poirier, L.J., Singh, P., Boucher, R., Olivier, A., and Larochelle, P., (1967).
Arch.Neurol., 17, 601-608

Poirier, L.J., and Sourkes, T.L. (1965)
Brain, 88, 181-192

Portig, P.J., and Vogt, M. (1968)
J.Physiol., 197, 20-21

Poschel, B.P.H., and Nintemann, F.W. (1966)
Life Sci., 5, 11-16

Powell, T.P.S., Cowan, W.M., and Raisman, G. (1965)
J,Anat.(Lond.), 99, 791-813

Powell, T.P.S., and Cowan, W.M. (1967)
J.Neurol.Neurosurg.Psychiat., 30, 140-153

Prado-Alcala, R.A., Kent, E.W., and Reid, L.D. (1975)
Brain Res., 84, 531-540

Precht, W., and Yoshida, M. (1971)
Brain Res., 32, 229-233

Pycock, C., and Horton, R. (1976)
Brain Res., 110, 629-634

Rausch, L.J., and Long, C.J. (1971)
Brain Res., 29, 146-150

Rebec, G.V., and Groves, P.M. (1975)
Neuropharmacology, 14, 275-282

Renaud, L.P. (1976)
J.Physiol.(Lond.), 260, 237-252

Roos, B.E. (1969)
J.Pharm.Pharmac., 21, 263-264

Roth, R.H., Murrin, L.C., and Walters, J.R. (1976)
Europ.J.Pharmac., 36, 163-171

Roth, R.H., Salzman, P.M., and Morgenroth, V.H. (1974)
Biochem.Pharmacol., 23, 2779-2784

Roth, R.H., Walters, J.R., Murrin, L.C., and Morgenroth, V.H. (1975)
in "Pre- and Postsynaptic Receptors", pp.5-46. Eds: Usdin, E., Bunney, W.E. Marcel Dekker. New York

Routtenberg, A. (1974)
in "Efferent Organisation and the Integration of Behaviour".
pp.264-318. Ed., Maser, J.D. Academic Press.

Routtenberg, A., and Holzman, N. (1973)
Science, 181, 83-86

Routtenberg, A., and Malsbury, C. (1969)
J.Comp.Physiol.Psychol., 68, 22-30

Scalia, F. (1966)
J.Comp.Neurol., 126, 285-310

Schwartz, W.J., Sharp, F.R., Grunn, R.H., and Evarts, E.V. (1976)
Nature, 261, 155-157

Scott, J.W., and Chafin, B.R. (1975)
Brain Res., 88, 64-68

Scott, J.W., and Leonard, C.M. (1971)
J.Comp.Neurol., 141, 331-344

Scott, J.W., and Pfaffmann, C. (1967)
Science, 158, 1592-1594

Scott, J.W., and Pfaffmann, C. (1972)
Brain Res., 48, 251-264

Seeman, P., and Lee, T. (1974)
J.Pharmacol.Exp.Therap., 190, 131-140

Seeman, P., and Lee, T. (1975)
Science, 188, 1217-1219

Sethy, V.H., Kuhar, M.J., Roth, R.H., van Woert, M.H., and Aghajanian, G.K., (1973)
Brain Res., 55, 481-484

Sharman, D.F. (1966)
Brit.J.Pharmac.Chemotherap., 28, 153-163

Sherlock, D.A., Field, P.M., and Raisman, G. (1975)
Brain Res., 88, 403-414

Shiebel, M.E., Schiebel, A.B., Mollica, A., and Morruzi, G.
J.Neurophysiol., 18, 309-331

Smaha, L.A., and Kaelber, W.W. (1973)
Exp.Brain Res., 16, 291-308

Snyder, S.H., Banerjee, S.P., Yamamura, H.I., and Greenberg, D. (1974)
Science, 184, 1243-1253

Spencer, H.J., and Havlicek, V. (1974)
Canad.J.Physiol.Pharmacol., 52, 808-813

Stadler, H., Lloyd, K.G., Gadea-Ciria, M., and Battholini, G. (1973)
Brain Res., 55, 476-480

Stricker, E.M., Friedman, M.I., and Zigmond, M.J. (1975)
Science, 189, 895-897

Stricker, E.M., and Zigmond, M.J. (1974)
J.Comp.Physiol.Psychol., 86, 973-994

Stricker, E.M., and Zigmond, M.J. (1976)
in "Progress in psychobiology and physiological psychology."
Vol.6, pp.121-187. Ed., Sprague, J.M. Academic Press.

Swaab, D.F., Pool. C.W., and Nijvelot, F. (1975)
J.Neural Transmission, 36, 195-215

Szabo, J. (1962)
Exp.Neurol., 5, 21-36

Szabo, J. (1967)
Exp.Neurol., 19, 463-476

Szabo, J. (1969)
Anat.Record, 163, 272-

Szabo, J. (1970)
Exp.Neurol., 27, 1-15

Szabo, J. (1972)
Exp.Neurol., 37, 562-572

Teitelbaum, P., and Epstein, A.N. (1962)
Physiol.Revs., 69, 74-90

Thierry, A.M., Blanc, G., Sobal, A., Stinus, L., and Glowinski, J. (1973)
Science, 182, 499-501

Trabucchi, M., Cheney, D., Racagni, G., and Costa, E. (1974)
Nature, 249, 664-666

Troiano, R., and Siegal, A. (1975)
Exp.Neurol., 49, 161-173

Tulloch, I.F. (1976)
Brit.J.Pharmac. (in the press)

Turner, B.H. (1973)
J.Comp.Physiol.Psychol., 82, 37-47

Ungerstedt, U. (1968)
Europ.J.Pharmacol., 5, 107-110

Ungerstedt, U. (1971(a)).
Acta physiol.scand., Suppl.367, 82, 1-18

Ungerstedt, U. (1971(b)).
Acta physiol.scand., Suppl.367, 82, 49-68

Ungerstedt, U. (1971(c)).
Acta physiol.scand., Suppl.367, 82, 69-93

Ungerstedt, U. (1971(d)).
Acta physiol.scand., Suppl.367, 95-122.

Ungerstedt, U. (1973)
in "Neurosciences Research", Vol.5, pp,73-96. Eds.: Ehrenpreis, S., and Kopin, I.

Ungerstedt, U., and Arbuthnott, G.W. (1970)
Brain Res., 24, 485-493

Ungerstedt, U., and Marshall, J.F. (1975)
in "Chemical Tools in Catecholamine Research." Vol.1, pp.311-318. Eds.: Jonsson, G., Malmfors, T., and Sachs, C.

Verhaart, W.J. (1950)
J.Comp.Neurol., 93, 425-440

Vogt, M. (1954)
J.Physiol., 123, 451-481

Voneida, T.J. (1960)
J.Comp.Neurol., 115, 75-87

Walters, J.R., Bunney, B.S., and Roth, R.H. (1975)
Advances in Neurology, 9, 273-284. Eds.: Calne, D.B., Chase, T.N., and Barbeau, A. Raven Press, New York.

Watson, W.E. (1968)
J.Physiol.(Lond.), 196, 122-123

Wedgewood, M. (1974)
J.Physiol., 239, p.88

Wise, C.D., and Stein, L. (1970)
in "International Symposium on Amphetamines and Related Compounds", pp.463-485. Eds.: Costa, E., and Garattini, S. Raven Press, New York.

Wise, R.A. (1976)
in "Brain-Stimulation Reward", pp.205-210. Eds.: Wauquier, A., and Rolls, E.T. Elsevier.

Wolf, G., and Sutin, J. (1966)
J.Comp.Neurol., 127, 137-156

Wong-Riley, M.T.T. (1975)
Anat.Rec., 181, 512

Wong-Riley, M.T.T. (1976)
Brain Res., 108, 257-278

York, D.H. (1972)
Brain Res., 37, 91-99

Yoshida, M., and Precht, W. (1971)
Brain Res., 32, 225-228

Zigmond, M.J., and Stricker, E.M. (1972)
Science, 177, 1211-1214

Zimmerman, E.A. (1974)
Endocrinology, 96, 1475-1477

Zis, A.P., Fibiger, H.C., and Phillips, A.G. (1973)
Science, 185, 960-963